Application of Different Measures of Bioavailability at Contaminated Sites

by

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Author’s Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

Contaminated areas resulting from anthropogenic activities have, for the most part, concentrations of contaminants that exceed Tier 1 standards below which the risk is considered acceptable. However, contaminants that have been in soil for a prolonged period can become recalcitrant over time, due to various physico-chemical and biological processes. Sequestered and recalcitrant contaminants are not readily biologically available to living organisms. However, they are easily measured analytically because of the strong acid extractions that are used in the analytical methodologies. Because toxicity is a function of exposure concentration(s), exposure duration, and bioavailability, contaminants in soil can be present at concentrations that exceed established standards but they represent minimal risk to ecological receptors because the contaminants are not fully available. To predict toxicity and estimate risk, it is imperative that an accurate and reliable measure of bioavailability be available.

Several surrogate measures of bioavailability were compared to the results of a battery of toxicity tests using Cu, Pb, and Zn-contaminated soils collected from a former industrial area and Cu and Zn-contaminated soils collected from a former mining site. $CaCl_2$ extractions, hydroxypropyl-β-cyclodextrin (cyclodextrin) extractions, Simulated Earthworm Gut (SEG) tests, and bioaccumulation tests were performed using the soils. Overall, SEG-extractable Cu was most predictive of adverse effects in industrial soils, likely due to enzymatic activity and/or increased ionic strength of the solution. For the mining soils, all chemical measures of bioavailability correlated with several biological responses; however, $CaCl_2$-extractable Cu and SEG-extractable Cu and Zn best predicted earthworm responses. Total Cu concentrations in soil correlated best with adverse effects to plants. No method was a good predictor of all biological effects for a single organism when data from the two sites were combined. The SEG test may provide a good indication of metal toxicity at contaminated sites with varying soil physico-chemical characteristics but further validation is required.

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Abbreviations

AS	Artificial soil
BAF	Bioaccumulation factor
BLM	Biotic Ligand Model
CBR	Critical body residue
CEC	Cation exchange capacity
DI	De-ionised
DTPA	Diethylenetriaminepentaacetic acid
EDTA	Ethylenediaminetetraacetic acid
GI	Gastrointestinal
ICP-MS	Inductively coupled plasma mass spectrometry
OECD	Organization for Economic Co-operation and Development
OM	Organic matter
PAH	Polycyclic aromatic hydrocarbon
SEG	Simulated Earthworm Gut
TBLM	Terrestrial Biotic Ligand Model
WHC	Water holding capacity

CHAPTER 1

INTRODUCTION

1.1. Metal pollution in soils

Metals do not degrade and are inherently persistent in the environment [3]. While metals are a natural constituent of both biotic and abiotic matter, localised metal concentrations in soil can become elevated to hazardous levels as a result of metal pollution. Metal pollution can result from anthropogenic activities such as mining operations, irrigation, sewage sludge application, pesticide use, industrial activities, and naturally from volcanic activity, geysers, meteors, *etc*. Areas contaminated with metals as a result of anthropogenic activities and identified as brownfields have, for the most part, concentrations of metal contaminants that exceed jurisdictional Tier 1 standards below which the associated risks to human health and/or ecological receptors are considered acceptable (i.e., minimal risk). Soil quality criteria and standards are typically based on total metal concentrations in soil. However, contaminants that have been in soil for a prolonged period can become "recalcitrant" over time, due to various physico-chemical and biological processes (e.g., ageing, weathering, sequestration, adsorption, degradation, *etc*.) [5-9]. Sequestered and recalcitrant residuals are not readily biologically available to human or ecological receptors. Ecological receptors including terrestrial plants and invertebrates, particularly earthworms, will be the focus throughout this thesis because they are good biological indicators of metal pollution in soils. The fate and behaviour of metals in soils is primarily metal dependent, and three metals are investigated herein: copper (Cu), lead (Pb), and zinc (Zn). These metals are investigated because they were present in the soils used in this thesis at total concentrations greater than provincial standards.

1.1.1. Copper

When applied to soils, Cu typically persists in the upper layers because it can be strongly bound to organic matter (OM) [10, 11]. It is associated with various components of the geosphere (i.e., adsorbed onto clay surfaces, complexed or incorporated by iron and manganese oxyhydroxides, present in the lattice of silicates, carbonates, phosphates, sulphates, and oxides, and bound to dissolved organic carbon) [8, 11-13]. Cu is an essential element that plays a vital role in cells and tissues (e.g., co-enzymes for metabolic pathways), and the minimum level of Cu required for biochemical function of earthworms is 8 mg/kg in soil [14]. However, Cu is also one of the more toxic metals to organisms including earthworms ([14] and references therein).

1.1.2. Lead

Like Cu, Pb is mainly bound to soil OM [12] and may be more tightly sorbed to soil particles than other metals such as cadmium (Cd) or Zn [15]. Due to this relatively strong sorption, Pb in soil may not be readily available for dermal uptake by soil invertebrates [16]. It is clear from toxicological studies that a Pb concentration in one soil type might be lethal to earthworms yet the same concentration in another soil type might have no detectable lethal or sublethal effects to the same species of earthworm [15]. Unlike Cu or Zn, Pb does not play a role in the survival of biota and is therefore considered a non-essential metal.

1.1.3. Zinc

Zn is an essential metal to soil organisms with important acid catalyst, control ion, and structural ion functions [17]. Zn is essential to the control of cell respiration and tissue growth, and in the development and regeneration of earthworms [18]. However, it can be toxic to most organisms when present in soil at high concentrations or when exposures are long [19]. A

comparison of toxicological data from laboratory and field studies revealed that of the four metals of concern (i.e., Cd, Cu, Pb and Zn) in soils located near a smelter, Zn had the greatest influence on earthworm distribution [6].

1.2. Ecological risk assessment

When contaminants in environmental media exceed Tier 1 benchmarks, further site-specific investigation might be warranted. Risk assessment is one of the available options to investigate the potential risks to human (human health risk assessment) and ecological (ecological risk assessment) receptors. The primary aim of ecological risk assessments is to predict the probability of exposure to contaminants, and the magnitude and extent of impacts to organisms associated with a contaminated site [1]. This is typically achieved by conducting an exposure assessment, using site-specific receptor characteristics and activity patterns, and a toxicity assessment, using available toxicity data (often culled from the literature). The results of the exposure assessment and toxicity assessment are integrated to characterise risk(s).

The awareness of the importance of including ecological receptors into risk assessments has increased over the past few decades. Terrestrial organisms such as plant and invertebrate species are in direct contact with contaminated soils for a significant portion of their lifetime, and can serve as vectors for contaminant transfer to higher trophic levels [20, 21]. In a baseline or screening level risk assessment, typically total measured soil concentrations at a site are compared to published screening values (e.g., those listed in Table 1.1), and if the measured values exceeds the screening values, it is assumed that the contaminants at that site potentially pose a risk to ecological receptors until further evidence is collected to demonstrate otherwise. If a risk assessment uses total metal concentrations in soil to assess the potential risks to receptors, risk might be overestimated since metal bioavailability is not taken into account [5, 22]. The accuracy of the estimates of risk produced via ecological risk assessment is

constrained by the myriad of conservative assumptions, one of which is that total metal concentrations in soil represent the “bioavailable” portion.

1.2.1. Role of earthworms in ecological risk assessment

Earthworm species are perpetually exposed to the soil environment and, as such, can accumulate metals from contaminated soils [23]. Some species of earthworms selectively feed on portions of soil that are rich in OM [24] and others are terrigenous (i.e., dirt eaters) [25]. Soft-bodied organisms such as earthworms can accumulate metals through dermal contact with pore water or ingestion of bulk soil or metal-contaminated organic material [15]. The accumulation of metals by earthworms can result in adverse effects depending on a number of factors, one of which is the type and nature of the metal. Because earthworms are so intimately exposed to soil at contaminated sites, they can serve as ideal indicator organisms in ecological risk assessment. As such, many soil benchmarks often are derived on the basis of the contaminant’s toxicity in soil to earthworms. Earthworms also serve as important vectors for trophic transfer of contaminants to higher levels, and contaminant doses in birds and vermivorous mammals are often modeled in ecological risk assessment based partly upon bioaccumulation of the contaminant by earthworms. Whether adverse effects manifest in an exposed earthworm or not, the worm serves as a significant food source for higher organisms, and metals may be subsequently accumulated and cause adverse effects in those organisms [21, 26, 27].

Table 1.1. Soil screening values (mg/kg) commonly used in ecological risk assessment.

Metal	Ontario Ecotoxicity Criteria [28]	Canadian Council of Ministers of the Environment [29]	United States Environmental Protection Agency Plant Eco-SSL [30]	United States Environmental Protection Agency Invertebrate Eco-SSL [30]	Oak Ridge National Laboratory Plant Benchmark [31]	Oak Ridge National Laboratory Invertebrate Benchmark [32]
Copper	225	91	70	80	100	50
Lead	n/v	600	120	1700	50	500
Zinc	600	360	n/v	n/v	50	100

n/v = No value

Eco-SSL = Ecological Soil Screening Level

1.3. Metal bioavailability

Bioavailability and bioaccessibility are fundamental terms germane to human and ecological risk assessment and many definitions of the words circulate in the literature. For clarification, the term “bioavailability” throughout this thesis refers to the amount of a contaminant (i.e., a metal) that is taken up, or can be taken up, by an organism from a specific environmental compartment (i.e., soil) from either direct contact (i.e., dermal uptake) or ingestion (i.e., oral uptake) [1, 15]. The term “bioaccessibility” refers to the portion of a metal that is solubilised from soil, following ingestion, and is mobilised in the gut fluids; therefore, it represents the maximum amount of a metal that can be taken up through the intestinal wall [1, 8]. The bioaccessible fraction does not take into account differential selective uptake across the intestinal membrane, nor does it take into account metabolism, sequestration, or secretion. Therefore, it provides a conservative estimate of bioavailability.

Typically, the total concentrations of metal present in a soil sample can be measured using non-selective analytical procedures such as hot concentrated acid extraction. Soil quality criteria and Tier 1 screening benchmarks including those in Table 1.1 are often derived as effects-based, total metal concentrations [15]. Test organisms are exposed for a specified duration to freshly spiked soil and a no effect or low effect benchmark is derived based on the response of the organisms as a function of the total concentration of contaminant in the exposure media. Usually, in these types of tests the compound is readily bioavailable. However, metals are rarely, if ever, 100% bioavailable in contaminated site soils [33]. Because toxicity is a function of exposure concentration(s), exposure duration, and bioavailability, contaminants in site soils can be present at concentrations that exceed established benchmarks (i.e., soil quality criteria, guidelines, or standards) but they can represent minimal risk to ecological receptors because the contaminants are not fully available [8, 9, 34].

The major limitation to establishing soil-quality criteria and standards using total metal concentrations is attributed to the profound influence that soil characteristics may have on metal bioavailability [14, 35]. Soil chemistry is complex and it is controlled, to a large extent, by adsorption-desorption interactions with solid surfaces, exchange processes with OM, and complexation with dissolved organic and inorganic ligands. Metals can exist in soil in the aqueous phase, adsorbed onto the surface of soil particles, or as precipitates [3]. Janssen *et al*. [36] indicated that cation exchange capacity (CEC), which is a measure of the number of available sorption sites, is one of the most important soil characteristics influencing metal bioavailability. CEC itself is influenced by soil pH, composition (i.e., fraction of clay, OM, etc.) and number of competitively sorbed ions [36]. Many studies have confirmed that varying these soil physico-chemical parameters alters metal toxicity to soil organisms [15, 35, 37-40]. However, Roembke [41] found toxicity of zinc nitrate-tetrahydrate in natural soils to earthworms was only weakly correlated with pH, organic carbon content, or CEC; increasing pH significantly decreased toxicity to *Folsomia candida*; and increasing pH and CEC marginally decreased toxicity to turnip rape. The authors attributed the lack of significant correlations observed between soil parameters and earthworm toxicity to the wide range of variability of the soil parameters in the natural soils tested, in comparison with artificial soils typically used by other researchers [41]. Soil parameters influence metal bioavailability by affecting the sorption of metal ions to soil particles, decreasing the concentration of free metal ion in solution by complexation, or by increasing competition at metal uptake sites [42]; therefore, metal bioavailability varies for different site soils with different characteristics. Using the total concentrations of metals in soil is inadequate for the prediction of effects to ecological receptors [7, 11, 14, 16, 33, 37, 42-45] and does not provide any indication of metal speciation or behaviour in site soils [22].

Significant efforts have been made in the last two decades to move from a total-metal based approach to a bioavailable-metal based approach to evaluate toxicity to ecological receptors, or to move up the "hierarchy of analyses" (Fig. 1.1). To predict toxicity and estimate risk, it is imperative that an accurate and reliable measure of bioavailability is available. Although conceptual models of metal bioavailability are useful, even more useful are actual direct or indirect measures of bioavailability, many of which were reviewed in Lanno *et al*. [15]. Other than modeling, there are two approaches that can be taken to measure bioavailability. Bioaccessibility tests and other biomimetic devices (semipermeable membrane devices (SPMDs)), biotic ligand modeling, and chemical extractions (e.g., calcium chloride, calcium sulphate, nitric acid, ammonium-ethylenediaminetetraacetic acid (EDTA), diethylenetriaminepentaacetic acid (DTPA), cyclodextrin) are surrogate chemical measures of bioavailability. Toxicity tests, measuring contaminant residues in tissues, and bioaccumulation tests are surrogate biological measures of bioavailability. Biological measures often use earthworms as test organisms, because they ingest significant amounts of soil and/or OM, and are also in constant contact with soil pore water. Each of these tools has advantages and limitations and has proven useful when applied within a specific research context. Some measures provide more accurate measures of the true bioavailability of metals in soil while others give little indication of the amount of a metal that is actually bioavailable. Regardless, there are no standardised methods for measuring bioavailability and no one method or suite of methods can be recommended over another because there are insufficient comparative or applied data [1, 15].

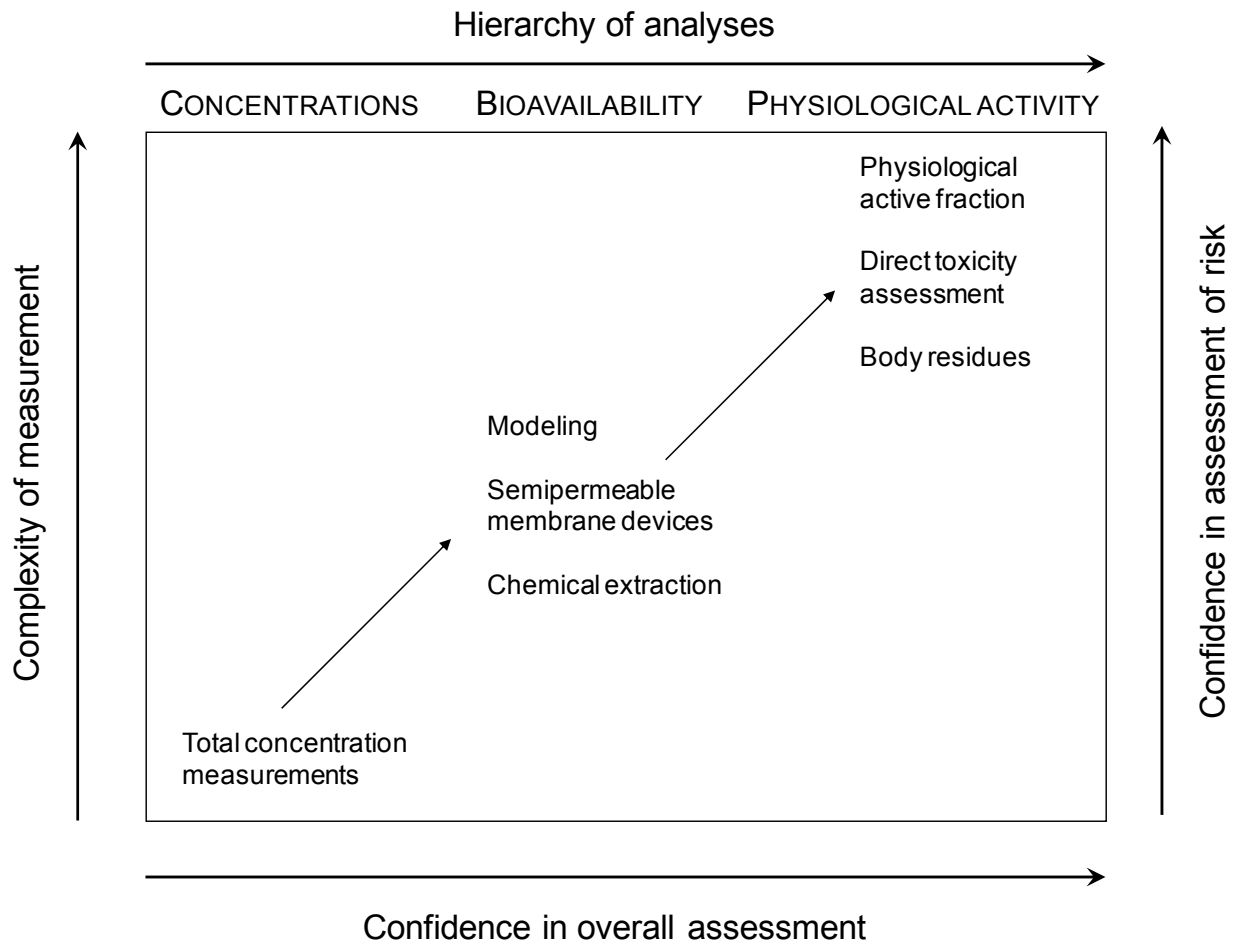

Fig. 1.1. A comparison of various measures of metal bioavailability in soil (modified from [1]).

1.3.1. Soil pore water

Pore water is thought to be either the main source of exposure for invertebrates and plants [15, 16, 46] to metals in soil, or at least a significant exposure pathway (i.e., an uptake route which is related to the soil pore water metal concentration) [46, 47]. Based on this understanding, many researchers have used an equilibrium partitioning approach to model the amount of metal solubilised in pore water based on the total metal concentration in soil and soil characteristics (e.g., [8, 40]). The equilibrium partitioning approach does not consider food as a possible route of uptake, and may not be a suitable model for predicting bioavailability to organisms whose primary route of uptake is through ingestion [8]. Some data suggest that soluble metal concentrations in pore water best describe bioaccumulation in earthworms [39, 48-50], but, ultimately, evidence of porewater uptake is circumstantial for metals [51]. Soluble metals are not necessarily bioavailable for invertebrate or plant uptake [22]. For some metals such as Zn, exposure through ingestion of soil by invertebrates may be a significant route of uptake [45, 52]. McLaughlin *et al*. [53] found that increased complexation of metals with inorganic or organic ligands actually increased metal bioavailability to plants. It is apparent that the influence that porewater concentrations have on metal bioavailability is both soil and contaminant specific, and the use of porewater concentrations alone to estimate metal bioavailability may be overly simplistic.

1.4. Modeling

Many researchers have developed models to describe the partitioning of metals from soil or sediment into pore water or biota (e.g., [40, 50, 54]). As suggested in Section 1.3.1, some equilibrium partitioning models such as OMEGA (optimal modeling for ecotoxicological applications) [45] do not consider ingestion of metals through soil as an uptake route for

invertebrates, although some evidence suggests that under specific circumstances this might be a major route of exposure. The significance of oral uptake on metal exposure is contaminant and site dependent. The use of these models may not accurately predict metal bioavailability at sites where a relatively large fraction of metal is sorbed to OM and not available via dermal uptake but could be ingested and mobilised in the gastrointestinal (GI) tract. For example, the OMEGA model accurately predicts Cd accumulation in earthworms; however, Cu, Pb, and Zn tissue concentrations were not accurately predicted [45]. Effort has been made to develop models to estimate metal bioavailability to earthworms that are not entirely dependent on pore water exposure. Saxe *et al*. [26] developed an earthworm model that incorporated dermal and gut exposure, recognising that the neutral pH of the earthworm's GI tract might release a different pool of soluble metals from soil. However, this approach does not account for metals released from soil particles by enzymes in the gut, only the pH effect. As well, this model was not validated due to a dearth of information in literature.

Any model that strives to reflect soil conditions must use soil characteristics to predict metal bioavailability. In their development of models to describe Cu toxicity to earthworms, Criel *et al*. [35] determined that although CEC best predicted Cu toxicity in field soils out of any one soil parameter, combining other soil parameters such as pH, clay content, manganese oxides, or aluminium oxides could better explain the observed Cu toxicity to invertebrates (likely because many of the soil parameters autocorrelate to some degree). Conversely, Janssen *et al*. [36] determined that dissolved iron was the most important variable governing the partitioning and bioavailability of Cu in site soils. Soil pH was the most important soil characteristic governing bioavailability of Pb and Zn. Cu and Zn bioavailability to plants was best predicted using models dependent on pH and OM, and pH alone, respectively [22], but Rooney *et al*. [55] observed that CEC was most influential on Cu toxicity to plants.

Perhaps the conflicting accounts of which soil characteristic(s) govern metal bioavailability in soils as determined from models is constrained by the soils and organisms used in their development. Models inherit the uncertainty associated with the assumptions and data used in their derivation [8]. Almost all soil models developed to date do not consider the presence of competing cations, yet the presence of cations such as sodium (Na), hydrogen (H), calcium (Ca), potassium (K), and magnesium (Mg) can significantly affect metal uptake and toxicity [3]. A biotic ligand model (BLM) has been used for several years to predict the fate and toxicity of inorganic compounds to aquatic receptors [56, 57]. In 2006, an equivalent theoretical model was developed by Thakali *et al*. [44, 58] for ecotoxicity assessment of metals in soils. The terrestrial (T) BLM concept takes into account not only metal activity but also the binding competition of competing cations. It assumes that the metal in soil is in equilibrium with the metal in soil solution, and the metal ions in solution bind to the biotic ligand on the organism to cause toxicity. The TBLM was demonstrated to be predictive of toxicity of Cu and nickel (Ni) to barley, tomato, soil invertebrates, and soil microbes in soils of a range of pH and organic carbon content [44, 58]. In theory, the TBLM should be applicable to all soils, a major advantage over previously reported metal bioavailability models reported in the literature. Although the TBLM is conceptually an attractive tool, its development is in the preliminary stages and it is not yet ready to be implemented in ecological risk assessment to the same extent as the aquatic BLM.

Unfortunately, a single model or set of models has not been developed and validated for a wide range of soils, and reports of the soil characteristics which have the largest impact on metal bioavailability are conflicting and are most likely site-specific. It would be useful if the regression models derived by independent researchers were tested with soils from different sites to determine applicability; however, this is often not the case. For those cases for which models were created and then validated using other soils, potential bioavailability is either over- or under-estimated [22]. Until biological regulation by organisms is somehow incorporated into

bioavailability models, it may be impossible to accurately predict bioavailability of essential metals such as Cu and Zn [45]. Rather than trying to transform total metal concentrations using a model to estimate the bioavailable fractions, it is more effective to express effect concentrations by a quantified bioavailable fraction related to toxic effects, such as those determined with chemical extractions [7].

1.5. Chemical measures

Extraction techniques other than those used in the determination of total metal content can provide a better indication of the bioavailable metal pool in soils [33, 47]. The most simple extraction solution one could use is de-ionised (DI) water. The results could, in theory, represent the porewater concentrations of metals which, as discussed previously, may be accurate predictors of metal bioavailability depending on the contaminant and soil type. However, this extraction solution is rarely used as it is often "too weak", the opposite of the argument that total metal extractions are "too strong" [12]. Alternatively, porewater concentrations could be measured directly from site soil, with no further addition of water to the soil sample. As one could imagine, measurement of pore water is a very intensive procedure that requires relatively large amounts of soil and the results are highly dependent on the method of extraction, the *g* force when centrifugation is used, and the moisture content of the soil [8]. In addition, concentrations in pore water extracts can lead to detection problems [36]. As measurement of porewater concentrations in soil can be a difficult process, some chemical extractions are often used as surrogate measures of porewater concentrations [3, 15]; some researchers consider chemical extractions as an intermediate between total metal determinations and porewater determinations [8] (i.e., somewhere between "too weak" and "too strong").

Bioavailability cannot be directly measured using chemical solutions, as only a biological organism can determine bioavailability to itself. However, several researchers have recognised that a method to estimate bioavailability using a chemical extraction would be an invaluable screening tool at contaminated sites (e.g., [33, 47, 59]). To develop a practical test for metal bioavailability, a chemical method must be validated with the bioavailable metal pool in the soil or some biological measure [15, 54]. If chemically extracted concentrations are determined for field soil and the same field soil was used to measure some biological response (i.e., toxicity), then one would be able to determine, indirectly, the bioavailability of the metal in soil [15]. Direct chemical measures of bioavailability are not currently available [15]. Validation of chemical measures with biological measures has been attempted numerous times with some success; Conder *et al*. [59] even suggested the possibility of developing universal incipient lethal levels (ILLs) for earthworms exposed to Zn based on $Ca(NO_3)_2$-extractable levels[1]. However, the authors identified the need to test other soils with different contamination issues and levels using weak salt extractions to further validate the concept of using chemical extractions to predict bioavailability. If an indirect chemical measure of bioavailability is repeatedly correlated with some direct measure of bioavailability using different metal concentrations and soil types, then theoretically it could be considered as a direct measure of bioavailability [15].

Typically, each chemical extraction method was developed with just one element in mind, and may not be applicable to all compounds in soil [8]. Numerous chemical extraction

[1] It is important to note that extractions can be either functionally-defined or operationally-defined, which may cause some confusion when comparing results from different studies. Throughout this thesis the results will be operationally-defined (e.g., "$CaCl_2$-extractable metal" as opposed to "mobile metal") where possible, because the use of functionally-defined terms is inconsistent in the literature and often vague.

methods are available, ranging from sequential extractions involving many reagents to simple procedures requiring only one reagent. Reports of the optimal extractant(s) for a particular metal in soil are conflicting: non-buffered salts and organic complexants are recommended as the "best" extractants [12], but $CaCl_2$, a non-buffered salt, and DTPA, a complexing agent, were neither useful nor consistent techniques in measuring Cu bioavailability to earthworms [47]. When Aten and Gupta [60] compared ten chemical extractants, some mixtures of complexing agents and weak salts, the difference between all extractants was marginal and all accurately predicted Cu and Zn tissue concentrations in ryegrass and lettuce. Quevauviller [61] raised the issue that the lack of standardised extraction methods makes interlaboratory comparison difficult, and the need for harmonization of procedures is strong.

It is recognised that no single extraction test can predict the bioavailability of all metals in all soils to all receptors. Extraction methods were developed with different aims, and some were initially developed to assess the nutrient availability to plants [1] before being adapted to assess bioaccumulation of metals (see Table 1.2). Various extraction techniques are reviewed in detail by Peijnenburg *et al*. [1] and are briefly discussed in the following sections. Along with sequential extractions, three broad categories of simple extraction tasks were identified [62]:

1. Extraction with ionised water or diluted acids;
2. Extraction with neutralised salt solutions; and
3. Extraction with complexing agents.

It is also common to see combinations of the extractions discussed below used in the literature.

Table 1.2. Weak chemical extractions diagnostic of plant uptake (modified from [4]).

Extractant	Metal	Correlated Plant Content
Water	Cd, Cu, Zn	Wheat, lettuce
0.05 M EDTA	Cd, Cu, Ni, Pb, Zn	Arable crops
0.05 M EDTA	Se, Mo	Greenhouse crops
DTPA	Cd, Cu, Fe, Mn, Ni, Zn	Beans, lettuce, maize, sorghum, wheat
2.5% acetic acid	Cd, Co, Cr, Ni, Pb, Zn	Arable crops, herbage
1 M ammonium acetate	Mo, Ni, Pb, Zn	Herbage, oats, rice, sorghum, Swiss chard
0.5 M ammonium acetate and 0.02 M EDTA	Cu, Fe, Mn	Wheat
0.05 M $CaCl_2$	Cd, Pb	Vegetable
0.1 M $NaNO_3$	Cd, Pb	Vegetable
1 M ammonium nitrate	Cd, Pb	Vegetable

1.5.1. Sequential extractions

Sequential extraction procedures employ two or more extractions in sequence to elucidate the operational fractions, based on decreasing solubility, of metals in soils. They are very useful for estimating the mobile and stable fractions of metals in soils. Perhaps the most widely used extraction procedures include the Tessier (five-step extraction) [63] or the Standards, Measurements and Testing programme (SM &T), formerly the European Community Bureau of Reference (BCR) (4-step extraction) (see [62] for references). Sequential extractions are primarily used for sediments [64], although they have been employed to determine partitioning of metals among soil compartments (e.g., [16]).

Sequential extraction procedures have been critiqued by various researchers (e.g., [1, 64-67]). The major disadvantages were summarised by Peijnenburg *et al*. [1]. Selectivity of the reagents are not perfect, and they may dissolve compounds other than those intended. In addition, metals solubilised in one step may subsequently re-adsorb in later steps (i.e. re-distribution among phases throughout the extraction), which will lead to an underestimation of bioavailability. As with most soil tests, sample handling prior to extraction can change speciation of metals and the results obtained in the laboratory might not necessarily be representative of field conditions. Another downside of sequential extractions pertains to the use of several extractants: with increasing number of extraction steps, the uncertainty associated with the test increases. Lastly, several pools of metals extracted in sequential extractions are often pooled in the end to relate to a bioavailable fraction, essentially reducing the entire process to a single extraction anyways [1].

Sequential extraction methods can be time consuming, and slight variations in extraction conditions such as pH can significantly affect reproducibility of the procedures [64].

Modifications that shorten the time needed to perform these sequential extraction methods [62], while successful, still require a considerable number of reagents and extraction steps.

1.5.2. Single extractions

Although some of the criticisms directed at sequential extraction procedures also pertain to single extractions (i.e., soil handling effects), use of single extractions is attractive because they are typically easy to perform and more cost-effective than sequential extractions [1]. As well, error is reduced as it is not magnified throughout subsequent extraction steps [1]. Only relatively fast, simple extraction procedures applying one extraction step that provides an estimate of bioavailable metals are used in this thesis. Single extraction procedures have produced comparable results in interlaboratory comparisons as long as technical requirements were strictly followed [61], which adds to their appeal as a universal extractant.

If a single extraction method is proven to be a reliable predictor of toxicity for one or several soil types and contamination issues, uncertainty will be reduced and reproducibility will be increased relative to sequential extractions, and the extraction procedure will be relatively simple for technicians to perform. In addition, costs of reagents will be less than those for more intensive sequential extraction methods, and the test can be completed in a matter of a few hours as opposed to up to 24 hours for other extraction methods.

1.5.2.1 Acid extractions

The most common acid extractions used to determine metal content of soils are those using concentrated strong acids such as HNO_3, HCl, HF, or aqua regia (HNO_3 and HCl). These extractions are often carried out at high temperatures using microwaves [8]. However, dilute solutions of these strong acids are sometimes used to estimate the bioavailable fraction of a metal in soil [1, 8]. For example, Ma [14] used a dilute HNO_3 solution to extract Cu adsorbed onto the soil matrix to estimate the potential amount available for uptake by plants and

invertebrates. Cu extracted with a 0.01 M HCl solution correlated better with lettuce, mustard, and barley uptake than Cu in weak salt and complexing agent extracts [22]. However, this was not true for Zn extracted from the soil.

Perhaps the more common acid extractions used to estimate bioavailability use weak acids such as acetic or citric acid or weak salts of acids such as ammonium acetate. These solutions have been used to assess Cd, Cu, and Pb bioavailability in soil to plants and invertebrates with marginal success [1, 11, 62, 68]. Weak salt solutions and complexing agents are more common for assessing metal bioavailability to ecological receptors.

1.5.2.2 Salt solution extractions

Extractions with salt solutions are usually better correlated with toxic effects than those with diluted acids or complexing agents [69]. Typical salt solutions used include $Ca(NO_3)_2$, $CaCl_2$, $MgCl_2$, $Sr(NP_3)_2$, NH_4NO_3, or $BaCl_2$ [1, 62]. Results for most mild salt extractions across a wide range of soils are correlated [51]. The $CaCl_2$ method is a commonly used, "soft" extraction method considered to represent the labile fraction of metals that has potential to enter terrestrial organisms (i.e., it provides an indication of the amount of metal that can be desorbed, or is water-soluble or exchangeable) [23, 39]. Calcium competes with absorption sites in soil particles [70]. The $CaCl_2$ extractable portion of some metals (e.g., arsenic (As), Cu) was a more accurate predictor of earthworm bioaccumulation (and hence bioavailability) than other extraction methods including the HNO_3 digestion commonly used to determine total metal concentrations [48]. However, total metal concentrations were more reliable than $CaCl_2$ extracts as indicators of the bioaccumulation of other metals (e.g., Cd, Cr, Zn), and neither method was a more reliable indicator than total porewater concentrations for the remaining metals analysed (e.g., Ni, Pb). Cd concentrations in earthworms were positively correlated to $CaCl_2$ extractable

portions of Cd in field soils [39]. $CaCl_2$-extractable Cu from soil correlated with toxicity to plants and invertebrates [38].

$CaCl_2$ concentrations of 1.0 M [70, 71], 0.5 M [38], 0.1 M [11, 12], and 0.01 M [7, 13, 14, 36, 39, 40, 47, 51, 72-74] have been used to estimate the bioavailability of metals in soils to ecological receptors. The most frequently used $CaCl_2$ extraction concentration of 0.01 M $CaCl_2$ was selected by Houba *et al*. [69] as the universal extractant to be used for a variety of purposes.

Cu extracted with 0.01 M $CaCl_2$ from soil correlated with porewater concentrations [7]. Although invertebrate and red clover toxicity correlated better with 0.01 M $CaCl_2$-extractable Cu than total Cu, red clover tissue residues were not predicted by $CaCl_2$-extractable Cu concentrations [7]. Extractable metals with 0.01 M $CaCl_2$ correlated best with the capacity of soils to supply Cd and Zn, but not Cu, to soluble metal pools [51]. These results suggest that accumulation of Cu could be influenced by bound Cu, and may not be entirely influenced by porewater concentrations. Extracting metals from soil at higher temperatures [73] or using higher concentrations of $CaCl_2$ [12, 71] could release a larger fraction of Cu that is bound to OM in the soil matrix, and provide a more accurate estimation of bioavailability to receptors exposed primarily to this pool of Cu. In addition, the use of a higher concentration of $CaCl_2$ could eliminate detection limit issues such as those observed by Spurgeon *et al*. [40], where Pb was not detected in 0.01 M $CaCl_2$ extracts. The influence of the $CaCl_2$ concentration used on the extraction of metals from polluted soils has been studied. Esnaola *et al*. [12] recommended a concentration of 0.1 M $CaCl_2$ over 0.01 M $CaCl_2$ for Cu-contaminated soils, but did not test higher concentrations. A concentration of 0.5 M was used in the $CaCl_2$ extractions carried out in this thesis based on preliminary testing using a range of $CaCl_2$ concentrations (i.e., from 0.01 M to 1 M, see Appendix A).

1.5.2.3 Extraction with complexing agents

Complexing agents such as DTPA, EDTA, or nitrilotriacetic acid (NTA) are expected to measure the mobilisable soil fraction (i.e., exchangeable and organically bound metals) [23]. DTPA and EDTA are chelating agents originally developed to determine micronutrient deficiency problems in soils but are also applied for other purposes including estimating bioavailable metals [47, 54, 73]. Often these complexing agents are combined with a weak salt or acid solution for extraction of metals in one step (e.g., [52, 72]). Generally, the metal concentrations extracted using these complexing agents alone are correlated with total metal concentrations in soil and not with biological measures of bioavailability [23, 38].

The hydroxypropyl-β-cyclodextrin (cyclodextrin) extraction method is a relatively new extraction method that has correlated well with bioavailable phenanthrene in soil irrespective of contaminant concentrations, soil pH and OM, or chemical contact time with soil [75, 76]. This compound is often referred to as HPCD in the literature, but will be referred to as cyclodextrin for ease of reference throughout this thesis. Cyclodextrins have high aqueous solubilities and hydrophobic interior cavities; they are thought to extract labile soil-bound organic contaminants without extracting sequestered molecules [75]. Cyclodextrin has been predictive of high-molecular weight polycyclic aromatic hydrocarbon (PAH) availability to freshwater worms (*Lumbriculus variegatus*) [77].

The cyclodextrin extraction is also a relatively simple and rapid method like the $CaCl_2$ extraction. So far, its application has been restricted to organic contaminants such as petroleum hydrocarbons (PHCs) and PAHs and it has rarely been used as a tool to measure bioavailability in soils from brownfields or contaminated lands. The extent to which this method can extract the bioavailable portion of metals in soils is unknown, but it could provide a good indication of organometallic compound bioavailability.

1.6. Biological measures

Biological measures of bioavailability determine the actual amount of metal taken up by an organism and provide the most accurate measure of bioavailability [15]. Biological measures such as bioaccumulation and ecotoxicity tests take into account the biotic and abiotic factors controlling metal bioavailability, as opposed to chemical measures, which generally only take into account abiotic factors. A comprehensive review of the use of soil fauna as pollution indicators is provided in Cortet *et al*. [78]. The most common biological measures of bioavailability include bioaccumulation and ecotoxicity testing.

1.6.1. Bioaccumulation tests

Bioaccumulation tests directly measure the metal concentration in an organism as a result of net influx from the soil, which is dictated by the rate of uptake and elimination of the metal [15]. Body residues are often better indicators of toxicity at a given site than total metal concentrations in soil, because inherent soil physico-chemical properties affecting bioavailability are accounted for [14, 15, 50, 79-81]. A similar concept is that of critical body residues (CBRs), which are the internal metal concentrations associated with toxic effects [15, 82]. The theory behind the CBR approach is that internal concentrations in the same species that cause toxic effects are the same regardless of the environment that the organisms are living in [83]. However, applying the CBR concept to metals (unlike organics) has proved problematic [79, 83], and body concentrations associated with toxicity may vary greatly in soil invertebrates [82] partially due to differences in within-organism metal compartmentalisation [79, 82]. Bioaccumulation tests can either take place in the laboratory under controlled conditions or in the field. For the latter, body burdens for organisms collected directly from a contaminated site are measured to provide an indication of metal bioavailability, but this does not account for avoidance behaviour or species' genetic resistance (adaptation) or acclimation.

Earthworms are the most commonly used organisms in soil invertebrate bioaccumulation studies. Uptake kinetics in ecophysiologically different earthworm species [23], and even closely related species [84] can differ significantly. This makes it difficult to select an appropriate test species when conducting bioaccumulation tests. Tissue residues have been measured in *Allolobophora* spp. [13, 21, 84, 85], *Aporrectodea* spp. [13, 18, 23, 39, 68, 84], *Dendrobaena veneta* [86, 87], *Dendrodrilus rubidus* [34], *Eisenia* spp. [6, 18, 23, 50, 70, 72, 88, 89], *Lumbricus rubellus* [13, 34, 39, 45, 84, 85, 90], *Lumbricus terrestris* [84, 91, 92], *Octolasium cyaneum* [93], and others to estimate bioavailability. *Eisenia* spp., the species recommended for ecotoxicity testing [17], have been used in the majority of accumulation studies [42]. *Eisenia* spp. are robust and easy to culture, mature in 8 weeks, reproduce at a higher rate than other earthworm species, and have a relatively short generation time [42]. For these reasons it is expected that *Eisenia* spp. will continue to be used in future testing despite some criticisms regarding their habitat (i.e., they are not a natural soil species) and sensitivity [42].

There is good evidence that accumulation of some metals may continue for the life span of the test organism [6, 83, 85, 90, 91]; hence, steady state cannot be reached in laboratory tests. A bioaccumulation factor (BAF) is often calculated at steady state as the ratio of metal concentration in the organism to the metal concentration in soil. Steady state is assumed when the concentration of metal in the organism has reached a “plateau” and does not continue to increase with exposure time. Alternatively, accumulation and elimination kinetics can be used to determine a kinetic BAF, as opposed to a BAF at steady state. In fact, there is a growing school of thought that accumulation rates, rather than absolute tissue concentrations, better predict metal toxicity [42]. Uptake characteristics have been successfully determined for several metals in earthworm bioaccumulation studies [6, 50, 83, 85, 94], although factors such as exposure time vary among studies.

No standardised ecological bioaccumulation test exists to date, although a draft test method has been proposed as an Organization for Economic Co-operation and Development (OECD) standard [95]. Tests conducted by Smith *et al*. [96] demonstrated that concentrations of Cd in artificial soils do not reach a steady state in *E. andrei*, consistent with results reported in other earthworm bioaccumulation studies [6, 50, 83, 85, 88]. The bioaccumulation and excretion of Zn in *E. andrei* did not follow a clear pattern in similar uptake and elimination experiments, perhaps due to the essentiality of the element, and the BAF was not calculated using kinetic parameters but instead with concentrations determined at the end of the uptake phase (e.g., steady state). The methodology used by Smith *et al*. [96] was suitable for determining uptake and elimination kinetics of a non-essential metal (e.g., Cd), and the steady state BAF for an essential metal (e.g., Zn).

Kinetic bioaccumulation testing is limited to metals that significantly bioaccumulate to a measureable level [15]. The challenge is to determine or define what constitutes “significant bioaccumulation”. The use of CBRs and bioaccumulation testing to predict toxicity of essential metals is hampered by factors such as hormesis and homeostasis which is influenced by the metal’s essentiality [14]. But essentiality does not necessarily determine whether a metal is bioaccumulated from the environment. While it is generally agreed that Zn does not significantly bioaccumulate in earthworms, the bioaccumulation patterns of Cu, which is an essential metal, are not established. It has been suggested that Cu does not reach steady state in earthworms regardless of exposure duration [13, 86]. Both Zn and Cu are essential for the normal functioning of earthworm physiological processes, and perhaps accumulation can be regulated to a certain threshold before internal body residues increase significantly with respect to those for unexposed worms. Non-essential metals such as Cd and Pb are typically bioaccumulated [6, 50, 68, 83, 85, 88]. Additional insight into the accumulation of essential metals such as Cu and Zn was identified as a future research need [45].

1.6.2. Ecotoxicity tests

Of all biological measures of bioavailability, the majority of the effort and attention has been dedicated to standardising and modifying protocols for conducting toxicity tests. Ecotoxicity tests were originally developed to assess the toxicity of soil and water media spiked with new and emerging chemicals [97]. Ecotoxicity tests provide a reliable estimate of acceptable soil concentrations [46]. Organisms are exposed both dermally and orally, which is essential in order to accurately interpret effects because metal bioavailability can be exposure route dependent [79]. Based on the responses of organisms used in ecotoxicity tests, researchers can conclude that the organism is affected by the bioavailable portion of metal in the soils (although not proven, the toxic effects are assumed to be from the soil contamination unless evidence suggests that other factors are hampering growth or reproduction) [15]. Quantifying the toxic effects of a metal in natural soils accounts for differences in soil characteristics such as pH, OM content, and CEC, but does not directly quantify the amount of metal that is bioavailable [15]. Results of bioassays are essential, however, to determine the predictive power of other measures of bioavailability through correlation [78]. In addition, tissue residues can be measured in test organisms to provide an indication of the CBR [8, 82, 94].

A large number of test methods exist for testing the toxicity of chemicals in soil to terrestrial plants and invertebrates, and are summarised in Roembke [97]. For this thesis, the standardised toxicity tests published by Environment Canada [98-100] for testing with plants, earthworms, and collembola were followed. The following subsections briefly describe the test species selected for use in this research.

1.6.2.1 Plant species selection

Twelve plant species are recommended by Environment Canada [98] for plant toxicity tests. Of these available species, a monocot and dicot species were selected for testing in

Chapter 2 and Chapter 3. Northern wheatgrass (*Elymus lanceolatus*) was used as a test species because it is a required test species in Tier 2 risk assessment in Alberta, Canada, and its sensitivity to PHCs and other contaminants has been demonstrated. It is widely distributed across North America and grows on a variety of soil types. Seeds have good viability and vigour in artificial and reference soils [101]. Alfalfa (*Medicago sativa* L.) and red clover (*Trifolium pratense* L.) were selected as the dicot test species in Chapter 2 and Chapter 3 respectively, based on their expected performance in the two soil types. For example, alfalfa grows well in loamy, well drained soils and it is tolerant to drought [101]. Red clover is more tolerant of water-logged soils with low pH [98]. Both dicot species are considered sensitive test species [101].

1.6.2.2 Oligochaete species selection

E. andrei (phylum, Annelida; class, Clitellata; subclass, Oligochaeata; order, Haplotaxida; superfamily, Lumbricoidea; family, Lumbricidae) is the preferred earthworm test organism in the laboratory at Stantec Consulting Ltd. and has been used in the majority of toxicity tests in-house. The benefits of using *Eisenia* spp. in laboratory testing were discussed in Section 1.6.1. Another type of worm used in standardised tests is the enchytraied (*Enchytraeus albidus* and *Enchytraeus crypticus*). Encyhtraieds are particularly amenable for testing some soils because they are more acid-tolerant than *E. andrei*. However, since no Environment Canada test method exists for testing with these animals, and because it was preferable to maintain the same Oligochaete species between tests, *E andrei* was used in testing with all soils herein.

1.6.2.3 Arthropod species selection

Collembola (phylum, Arthropoda; subphylum, Pancrustacea; superclass, Hexapoda) are microarthropods with an average body length of 1-5 mm [2]. Collembola are abundant

throughout the world, and approximately 7,500 different species have been identified [2]. *F. candida*, *Folsomia fimetaria*, and *Orthonychiurus folsomi* are commonly used in bioassays in most laboratories including that of Stantec Consulting Ltd. *F. fimetaria* are abundant in agricultural soils but may not inhabit forest soils rich in OM, and the distribution of *O. folsomi* is unknown but they are present in North American soils. *F. candida* can be found in agricultural soils but are most commonly found in flower beds or other anthropogenically impacted soils [97, 102]. Although it is not typically abundant in field soils, it is the collembolan species most often used in terrestrial toxicity tests [97], because it reproduces parthenogenically and is relatively easy to culture [2]. It feeds primarily on dead OM but also fungal mycelia, nematodes, and bacteria [2]. *F. candida* was recently recommended as a potential test species for the testing of soil from Canadian boreal forests and northern lands [102], which are high in OM content. Neither *F. fimetaria* nor *O. folsomi* were recommended as test species for those types of soils. The use of predatory mites such as *Hypoaspis aculeifer* for ecotoxicity testing is promising but no method has been standardised for use in Canada. Therefore, *F. candida* was used as the arthropod test species for this research.

1.7. Simulated Earthworm Gut

The Simulated Earthworm Gut (SEG) was developed in collaboration with researchers from the University of Saskatchewan, Saskatoon, Saskatchewan, Canada, and aims to mimic the conditions of the GI tract of *E. andrei* to determine the bioaccessibility of contaminants in soil. The following subsections will briefly describe the theoretical foundation of the test and the preliminary testing that occurred during the development of the SEG test. For more details, please consult Ma *et al*. [103].

1.7.1. Theoretical foundation for the SEG test

Earthworms colonise numerous terrestrial biomes and play a significant role in cycling of nutrients and structuring the soil environment. They live within the soil layer and ingest significant amounts of soil relative to their body size [92]. Thus, exposure to soil contaminants may be through the dermal or oral route, and the primary route of uptake is dictated by several factors including soil characteristics, contamination type, contaminant speciation, and earthworm species. The bioaccumulation of metals (Cd, Cu, and Zn) in spiders, whose primary exposure route is through food/prey ingestion, was dependent on dietary differences [81]. Cd and Zn concentrations in earthworms (*L. terrestris*) with their mouths sealed (i.e., exposure was only through the dermal route) were 83% and 79%, respectively, of concentrations detected in *L. terrestris* able to ingest contaminated soil (i.e., exposure was through both dermal and oral uptake) [104]. In addition, Saxe *et al*. [26] estimated that 96% of total Cd and Cu uptake and 82% of total Zn uptake occurs through the dermal route using modeling procedures. Conversely, emphasis on the role of the gut in metal uptake in earthworms has been reported in other studies [16, 84, 105]. Ultimately, the relative importance of dermal and ingestion exposure routes is dependent on the bioavailable metal concentrations in the soil solution and the gut [39]. It is possible that metals strongly bound to OM in the food of earthworms are taken up in the gut and this uptake would not be accounted for by chemical extractions that estimate porewater concentrations [104].

For some metals, it is widely known that the fraction of a metal in soil pore water is predictive of dermal uptake by earthworms. To estimate the labile fraction, chemical extractions as described in Section 1.5 are commonly used. However, at some sites concentrations of metals may be low in pore water and thus not readily available for dermal uptake. At such sites it is possible that the primary route of metal uptake is through absorption through the GI tract, since fractions of these metals unavailable in the external environment may be solubilised in the

GI tract with the aid of the endemic microorganisms. Therefore, bioaccessibility tests may correlate better with toxic endpoints if soil characteristics and metal type and form dictate that oral uptake is prominent, whereas chemical extraction tests may show better correlation if dermal exposure plays a significant role in metal uptake. Until now, no procedure has been developed to estimate the amount of metal solubilised from soil in an earthworm's GI tract (i.e., the bioaccessible fraction).

1.7.1.1 Eisenia andrei

E. andrei is one of the most commonly used earthworm species in earthworm ecotoxicity and bioaccumulation testing. *E. andrei* is an epigeic species (i.e., it primarily lives in compost and leaf litter as opposed to mineral soils) [2]. Despite its rarity in polluted soil environments typical of brownfield sites, it is ideal for use in laboratory testing because of its reproduction rate, relative population stability, and ease of culturing. It is generally believed to be a good surrogate for other, more predominant earthworm species in the environment. Since it is commonly used in laboratory testing, and was used for the ecotoxicity and bioaccumulation tests completed herein, the SEG test was developed to mimic, where possible, the conditions of the gut of *E. andrei*.

The main components of *E. andrei*'s alimentary canal are indicated in Fig. 1.2. Soil and OM enter through the mouth and are immediately amended with mucus that contains an amylase and a protease [2]. The pH of the esophagus is maintained near neutral. Food and soil are subsequently ground in the gizzard and passed to the intestine. Food is digested in the intestinal compartment by enzymes secreted by the endothelium of the earthworm gut and by microorganisms that are either ingested or reside in the gut. Undigested food and soil collect in the hind-gut are excreted through the anus [2] as cast.

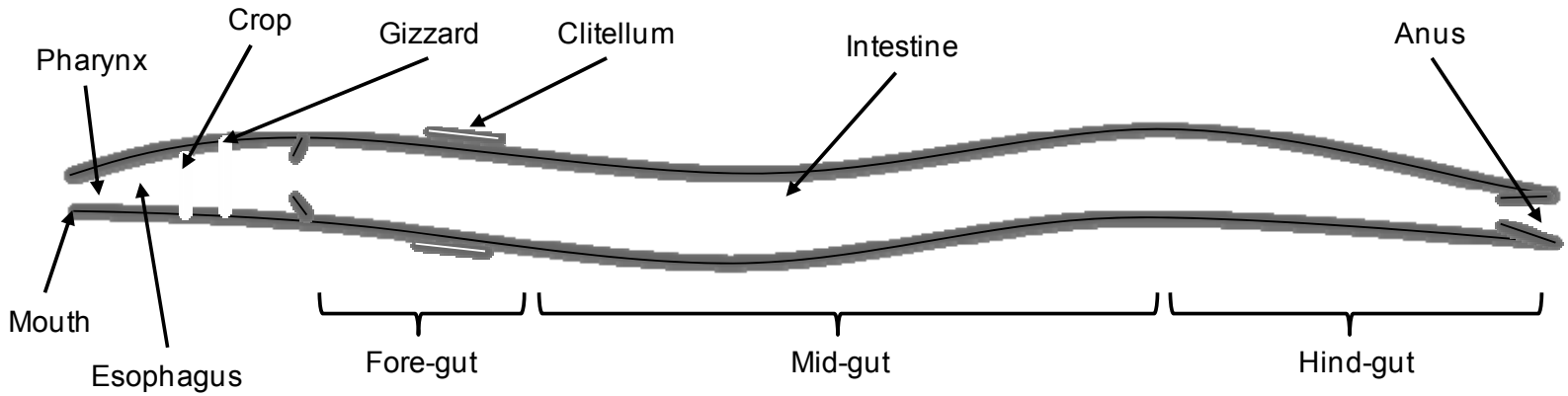

Fig. 1.2. The digestive system of *Eisenia andrei* (modified from [2]).

1.7.1.2 Earthworm gut composition

Many, if not all, earthworms contain endemic microorganisms within their GI tract. However, it is quite likely that due to the highly diverse microbial community that exists in most soil environments, the microbial composition of *E. andrei*'s GI tract is determined primarily by the soil that it ingests [2] and could be site-specific. *E. andrei* secretes mucus from the pharynx and calciferous glands that directly aid in the digestive process. However, this mucus also indirectly aids in the digestive process by serving as an energy source for microorganisms living in the gut. Thus, the relationship between *E. andrei* and its gut microbes is mutually beneficial: the earthworm gut environment encourages the growth of microorganisms by providing an energy source and hospitable environment, while the earthworm itself benefits through the additional help in breaking down OM.

The earthworm gut is an anoxic environment [106, 107] at a pH of near neutral, maintained primarily through the excretion of calcium carbonate in the gut mucus. Numerous enzymes have been identified in the earthworm's gut, including amylase, lipases, chitinase, cellulase, protease, peroxidase, and phosphatase [108-110]. However, it is nearly impossible to distinguish which of these enzymes is secreted solely by *E. andrei* itself, the gut microbes, or both [111]. However, for the purposes of designing this technique, the source of each enzyme (i.e., *E. andrei* versus microbes) is unimportant so long as the enzymes have been confirmed to be present in the gut environment.

1.7.2. Development of the SEG test

Before developing the SEG test, various human health bioaccessibility tests were researched. Bioaccessibility testing has been used in human health risk assessment for several years to estimate the bioaccessible fraction of contaminants in the human GI tract. Methods such as the Simulator of the Human Intestinal Microbial Ecosystem (SHIME) [112] and the *In*

Vitro Gastrointestinal (IVG) test [113], and the methodology from the Netherlands (RIVM) [114], utilise enzymes, salts, and biles to represent the stomach and/or intestinal (and sometimes an additional mouth/esophagus) compartment of a human and have been used to evaluate the bioaccessibility of metals (primarily As, Cd, and Pb) and PAHs. However, other methods such as the Relative Bioaccessibility Leaching Procedure (RBALP) [115] and European Standard Toy Safety Protocol (summarised in [116]) use a "simpler" approach—contaminants in soil are solubilised in solution of low pH (~1.5). The solutions do not contain complex mixtures of enzymes or bile. Extensive validation of these methods has occurred for some contaminants, and the RBALP procedure has recently been endorsed by the United States Environmental Protection Agency (US EPA) [117] to estimate bioaccessibility of Pb in soils to humans. It is important to note that although the RBALP correlates extremely well with *in vivo* studies of Pb bioavailability, it is by no means a surrogate human GI tract. This suggests that creating an exact representation of *E. andrei*'s GI tract may not be essential to predict metal bioavailability (as long as it correlates with toxicity).

Two approaches were investigated in the development of the SEG: a microbial and enzymatic approach. These are described in detail in Ma *et al*. [103]. Preliminary testing and method development indicated that the enzymatic solution was a more efficient extractant than the microbial solution. In fact, the microbial solution was comparable to extractions carried out using a weak (0.01 M) $CaCl_2$ solution and some metals were not detectable in some soil extracts. An extraction containing both microbial solution and enzymes was compared to each of extractions using only the microbial solution and enzyme solution. The microbial+enzyme solution was no more efficient at extracting As, Cu, Pb, and Zn than the enzyme solution alone.

The enzyme solution was the most promising for potentially mimicking the GI tract of *E. andrei*. The SEG test is a relatively easy procedure to carry out in the laboratory and does not require the continuous maintenance of microbial cultures in a chemostat. This procedure

was chosen for further testing and a more detailed description of the modified methodology is provided in Chapter 2 and Chapter 3. It is important to note that although the procedure used by Ma *et al.* [103] was generally followed, additional enzymes (i.e., phosphatase and trypsin), were used for the testing herein.

1.8. Scope of thesis

The soil contact exposure pathway for ecological receptors can be the main driver of ecological risk assessments. There is currently no standard method to measure bioavailability of metals in soil to ecological receptors, yet the influence of metal bioavailability on ecotoxicity has been known for decades and is a major issue in ecological risk assessment. Bioavailability can be drastically different at different sites with different characteristics [54]. The processes of metal partitioning in soil, interactions at the biological interface, organism uptake, sequestration, and toxicity have been reviewed [1, 3]. A simplified diagram of the fate of metals in soils and organisms is provided in Fig. 1.3. The portion of metals in soil that is available to partition through membranes of organisms is governed by factors such as pH, soil OM content, and the presence of other cations such as calcium. The portion of metals in soil that partition through membranes of organisms can be metabolised and/or excreted, accumulated in tissues, or transported to the site of toxic action [15, 82] (represented by “Organs” in Fig. 1.3). All of the internal processes may depend on the route of primary uptake [82].

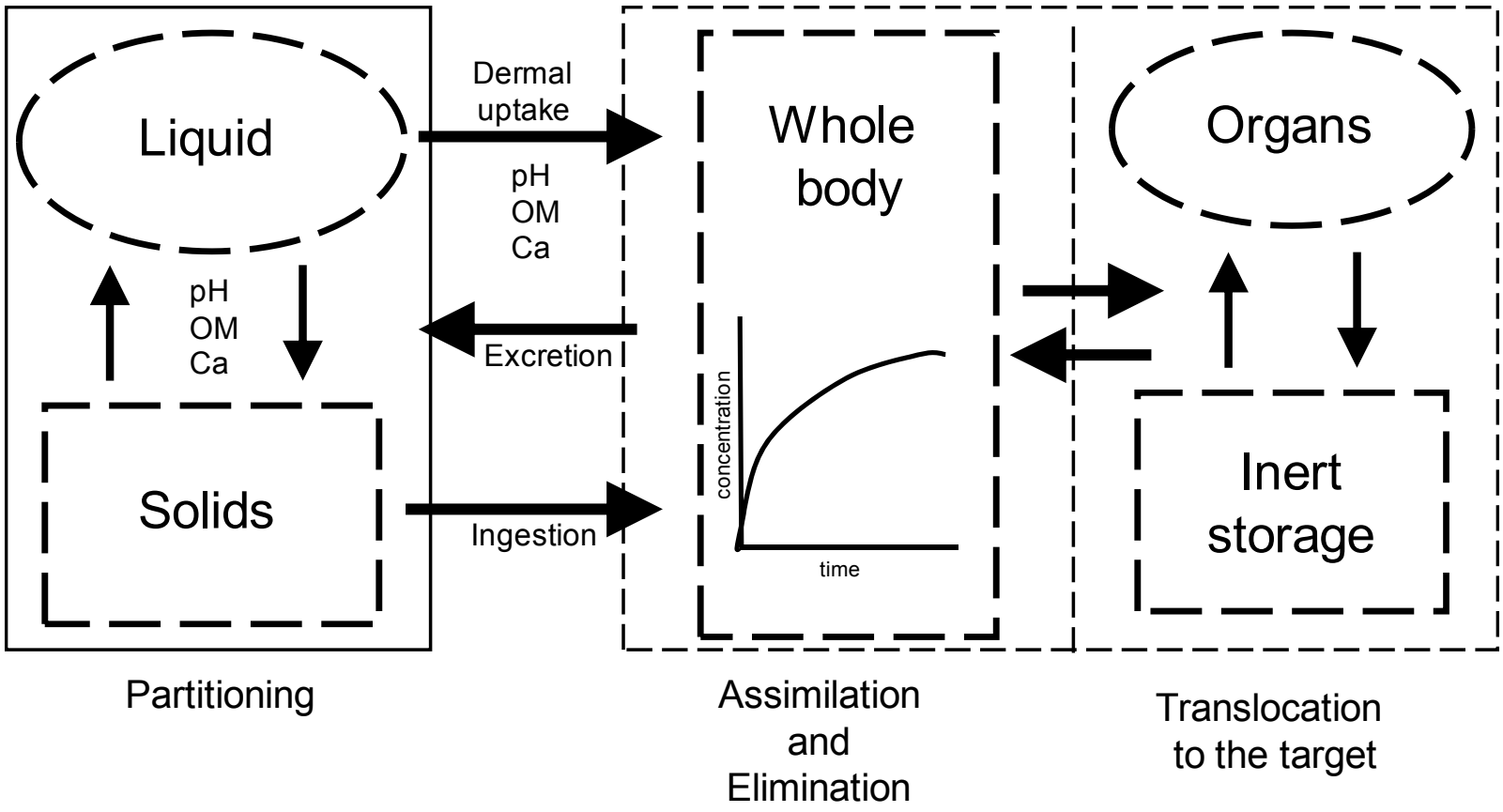

Fig. 1.3. A simplified diagram of the various factors controlling metal bioavailability and toxicity to ecological receptors (modified from [3]).

Currently, soil quality benchmarks and standards are based on the total concentration of the metal. The comparison of the total concentrations of metals at a brownfield site to these benchmarks typically represents a worst-case scenario. Assuming 100% bioavailability is an option that introduces a degree of conservatism that might be desirable from a precautionary position, but may neither be realistic [33] nor useful in terms of selection of site management options and decision making. Directly measuring the bioavailability of contaminants to ecological receptors mitigates some of the uncertainty currently associated with this assumption.

Researchers have investigated several methods of predicting metal bioavailability in soils, some of which were introduced in this Chapter. Soils at contaminated sites generally are heterogeneous with respect to their physico-chemical characteristics and they often contain complex mixtures of contaminants. Therefore, it is likely that bioavailability can be measured more accurately by applying tools tailored to the contamination and soil type at the site. The challenge is to determine which measurement tool is the most appropriate for which soil type and which contamination issue.

The advantage of successfully measuring bioavailability at a contaminated site or brownfield is that site-specific remedial objectives can be established that are technically achievable and less stringent than those comprising the Tier 1 standards, yet equally protective of the environment. Using a tool to measure bioavailability precludes defaulting to the assumption that a contaminant is 100% bioavailable. Understanding the advantages and constraints associated with the different tools for measuring bioavailability will result in the use of the most appropriate tool for a particular site (physico-chemical characteristics or site conditions) or contamination issue (type, magnitude and extent of contamination).

It is clear that the fields of ecological risk assessment and ecotoxicology lack information that relates measures of bioavailability to toxicological endpoints. The goal of this thesis was to

determine the bioavailability of metals (Cu, Pb, and Zn) by applying different laboratory tests to contaminated soils and to compare the results of each test. This will begin the validation process with measures of bioavailability, including the SEG test, to provide critical information for future research in the field.

Soils were collected from two different sites, each having distinct characteristics. The bioavailability of Cu and Zn (and sometimes Pb) in these soils was measured using $CaCl_2$ extractions, cyclodextrin extractions, bioaccumulation tests, a battery of toxicity tests, and SEG tests. The results of the tests rely in part on chemical measures of the analytes in the different substrates (tissues, solutions, and soils). Each measure of bioavailability was compared using univariate and multiple regression procedures to the results of ecotoxicity tests to determine which measure was most highly correlated with effects. The five tests used are all considered to be surrogate measures of bioavailability. Each can be used to support risk assessment through the modification of exposure scenarios, determination of trophic transfer, determination of toxicity, and to predict risks.

This thesis is organised into four Chapters, including this introductory Chapter. Chapter 2 details the results of the application of the different tools that were used to measure bioavailability of metals in soils collected from a former industrial area in Toronto, Ontario, Canada. Chapter 3 details the results of these tests applied to soils collected from a former mining site near La Sarre, Quebec, Canada. Finally, the implications of the results of both Chapter 2 and Chapter 3 are discussed in Chapter 4 in the context of their use for risk assessment. Chapters 2 and 3 were written as manuscripts for submission to a peer-reviewed journal. Although modified in form and content for publication, they still contain individual introduction, methods, results, and discussion sections.

CHAPTER 2

A COMPARISON OF MEASURES OF BIOAVAILABILITY IN SOILS FROM A FORMER INDUSTRIAL AREA

2.1. Introduction

Soil pollution is a major environmental concern as it devalues an important, irreplaceable resource. Contamination of soil with metals such as Cu, Pb, and Zn can adversely impact the environment, including ecological organisms living within the soil or on the soil surface. Tier 1 benchmarks are soil standards protective of the environment and used to identify regions or areas that require detailed assessment. However, Tier 1 ecological benchmarks may overestimate actual risks because they are often derived using data from the literature that were generated for other purposes. Frequently, the toxicity data were generated using soil freshly spiked with contaminants and not subjected to physical processes such as ageing, weathering, and sequestration that occur in the natural environment [5, 6, 8, 9, 22]. Therefore, if the total concentration for a metal in soil at a site (i.e., the amount liberated using a chemical extraction such as concentrated HNO_3) exceeds a Tier 1 value, site-specific investigation could be triggered to determine the risk associated with the metal in soil. The investigation could indicate that the Tier 1 assessment is correct and action is warranted, that the risk is overestimated, or that the risk is underestimated.

Conducting a risk assessment is a viable option for site-specific investigation. The actual risk to ecological receptors from exposure to a metal in soil hinges on the bioavailability of that metal. In ecological risk assessments, bioavailability values gleaned from the literature are sometimes used to estimate risk. These values are usually generated for species and substrates other than those that are representative of the soils under investigation, and are often calculated values based on literature data generated for purposes other than the intended use reflected herein. The uncertainty of using such literature values for establishing risk-based or remedial benchmarks is high. Still, the use of literature bioavailability values, despite the high uncertainty, is probably more representative of environmental conditions than defaulting to the assumption that the contaminants in soil are 100% bioavailable. Directly measuring the bioavailability of contaminants to ecological receptors mitigates many of the uncertainties.

There are two approaches that can be taken to measure site-specific bioavailability: chemical and biological. An overview of these two approaches was provided in Chapter 1, and all measures have their inherent strengths and weaknesses and have proven useful when applied within a specific research context.

Weak $CaCl_2$ extractions have been used for decades to estimate metal bioavailability. $CaCl_2$ extractions are commonly used, “soft” extraction methods considered to represent the labile fraction of metals that has potential to enter terrestrial organisms (i.e., it provides an indication of the amount of metal that can be desorbed from soil) [23, 39].

Cyclodextrins are thought to extract labile soil-bound organic contaminants without extracting sequestered molecules [75]. Although cyclodextrin has been used to extract PAHs [75, 76], it has not been used to estimate bioavailability of metals from contaminated soils. The extent to which this method can extract the bioavailable portion of metals in soils is unknown. Cyclodextrins are comprised of 6, 7, or 8 D-glucopyranonsyl residues linked by α-1,4 glycosidic bonds. Hydroxyl groups are located on the outer surface of the molecular cavity while the inner

cavity is lined with ether-like anomeric oxygen atoms and carbon and hydrogen atoms. It is possible that due to its hydrophobic organic interior, cyclodextrin may be more efficient than weak salt extractions at extracting organometallic forms of metals from soil.

The SEG test was recently developed based on the expected enzymatic composition of *E. andrei*`s gastrointestinal tract. Both a microbial approach and an enzymatic approach were investigated during the development of the SEG test, and the enzymatic approach was used for this thesis.

A bioaccumulation test with earthworms can measure the portion of metals in soil that is taken up and eliminated by the earthworm over time and the amount that is bioavailable for uptake is determined essentially on a mass balance basis. The bioaccumulation kinetics of some metals such as Cu are not well-defined in the literature. Kinetic bioaccumulation tests are preferred for determining the bioaccumulation of metals that do not reach steady state in earthworms within the test period.

Toxicity testing, although the most commonly used tool to measure site-specific bioavailability and toxicity, can be a costly and lengthy undertaking. Other techniques exist that aim to measure bioavailability, and thus estimate toxicity, that are relatively quick and inexpensive. The goal of the research comprising this chapter was to perform chemical extractions with $CaCl_2$ and cyclodextrin, the SEG test, and bioaccumulation tests, and to compare the results of these chemical and biological measures with those of a battery of toxicity tests. The correlations between the estimates of bioavailability and the toxicity data can provide an indication of which method shows the most promise for predicting site-specific bioavailability.

2.2. Methods

2.2.1. Site characteristics and soil collection

Field soils were collected near an urban park (Cherry Beach) in Toronto, Ontario, Canada. Historic lake filling practices at the site led to highly heterogeneous soil contaminated with metals. Seven bulk soil samples collected from the Site and an eighth soil sample collected off-Site were thoroughly homogenised and analysed for total metal content. The soil collected off-Site (i.e., soil A) was used as a reference soil representing an unimpacted area. Based on the results of these analyses, four of the contaminated soils were selected for use because they represented a range of metal contamination levels (i.e., soils B, C, D, and E). Metals of concern were Cu, Pb, and Zn, because they exceeded provincial soil quality guidelines in some of the site soils.

Artificial soil (AS) was formulated based on Environment Canada test methods by thoroughly mixing constituents in the following percentages (all values listed as % of soil dry weight): *Sphagnum* peat (Canadian HydroGardens Ltd., Ancaster, Ontario, Canada), 10%; silica sand (Optima Minerals, Waterdown, Ontario, Canada), 70%; and kaolinite clay (Tucker's Pottery Supplies Inc., Richmond Hill, Ontario, Canada), 20%. The moisture content of the AS was adjusted to approximately 28.5% on a dry weight basis by addition of DI water. $CaCO_3$ was sieved and added to the AS to adjust the final soil pH to 6.0 ± 0.5. The maximum water holding capacity (WHC) and physico-chemical characteristics of the site soils and AS were measured. WHC capacity was determined by saturating 100 g of dry soil with DI water, allowing the wetted soil to sit in a covered glass funnel, and weighing the wet soil after three hours of draining [98-100]. Physico-chemical analyses were performed by the Soil Nutrient Laboratory at the University of Guelph, Guelph, Ontario, Canada.

2.2.2. $CaCl_2$ extraction

$CaCl_2$ extractions were performed in triplicate for each site soil following methods outlined in Houba *et al*. [118], with modifications based on preliminary testing with the site soils. $CaCl_2$ was purchased from Thermo Fisher Scientific, Ottawa, Ontario, Canada. Preliminary extractions were performed using a range of literature reported $CaCl_2$ concentrations (from 0.01 M $CaCl_2$ solution to 1 M $CaCl_2$) to determine the optimum $CaCl_2$ concentration so that metal concentrations in extracts exceeded analytical detection limits (see Appendix A). A concentration of 0.5 M $CaCl_2$ proved to be sufficient and was used for the subsequent definitive soil extractions.

Field soils were sieved to ≤2 mm and air-dried for >72 hours. 10 ± 0.05 g of each soil were shaken at 120 rpm with 100 mL of the $CaCl_2$ solution for 2 hours in high-density polyethylene (HDPE) centrifuge tubes on a rotary platform shaker. Tubes were removed and centrifuged at 1,800 g for 10 minutes. This centrifugation step did not fully separate the soil from the solution, as suspended colloids were visible in the supernatant. Therefore, the supernatant of each tube was extracted using 0.45 µm cellulose acetate filters attached to disposable 20-cc syringes and filtered into clean
15-mL centrifuge tubes. Each 10-mL extraction fluid sample was acidified with 0.1 mL of 1 M HCl and stored at 4ºC. $CaCl_2$ solution spiked with known amounts of metal salts, as well as, unspiked $CaCl_2$ solution were submitted along with the extracts to ALS Laboratory Group in Waterloo, Ontario, Canada for metal analyses within one week of extraction.

2.2.3. Cyclodextrin extraction

Metal extractions were performed in triplicate on the site soils using a 0.035 M (40 g/L) cyclodextrin solution. Cyclodextrin was purchased from Sigma-Aldrich Co., Oakville, Ontario, Canada. Similar to the $CaCl_2$ tests, preliminary extractions were carried out to determine the

optimum cyclodextrin concentration; in addition extraction times ranging from 0.5 hours to 24 hours were used to determine their influence on extraction efficiency. From the preliminary tests, the cyclodextrin concentration and extraction time was chosen for definitive testing (see Appendix A).

5 ± 0.01 g of sieved (≤2 mm) air-dried site soil were shaken with 50 mL of cyclodextrin solution in HDPE centrifuge tubes for 5 hours on a rotary shaker at 120 rpm. The supernatant was removed from each tube using 20-cc disposable syringes and filtered through 0.45 µm cellulose acetate filters into 15-mL centrifuge tubes. Sample acidification, storage, time to analyses, and quality assurance procedures were as described for the $CaCl_2$ procedure.

2.2.4. Simulated Earthworm Gut extraction

The SEG extraction was carried out based on the results of preliminary testing that investigated several approaches. The development of the SEG extraction was thoroughly described by Ma *et al*. [103]. For these tests, only the enzymatic approach was used.

For each site soil, 2 g of sieved (≤2 mm), air-dried soil were added to a 15-mL plastic centrifuge tube, in triplicate. To each tube, α-amylase (from *Aspergillus oryzae*), cellulase (from *Aspergillus niger*), phosphatase (alkaline, from bovine intestinal mucosa), and trypsin (from porcine pancreas), dissolved in 4 mL of DI water, were added to obtain activities of 675 U, 186 U, 37 U, and 250,000 U, respectively. One unit (U) is the amount of enzyme activity which will catalyze the transformation of 1 micromole of substrate (enzyme-specific) per minute under standard conditions. All enzymes were purchased from Sigma-Aldrich Co., Oakville, Ontario, Canada. Tubes were placed on a rotary shaker and mixed at 210 rpm for 3.5 hours. Following mixing, tubes were centrifuged at 7,000 g for 20 minutes and filtered through 0.45 µm cellulose acetate filters attached to disposable 20-cc syringes into clean 15-mL centrifuge tubes. Sample

acidification, storage, time to analyses, and quality control samples were similar to those described for the other extractions.

2.2.5. Earthworm bioaccumulation test

The uptake phase of a 21-day earthworm (*E. andrei*) bioaccumulation test was performed following draft OECD guidance [95]. On day 0 of the test, each site soil and AS were moisturised to approximately 60% of the WHC and amended with oatmeal (as a food source) to a nominal concentration of 5 g/kg. Soils were allocated to 125-mL glass test vessels such that soil depth was approximately 5 cm (approximately 50 g soil dry wt.). Sexually mature earthworms, weighing approximately 400 mg wet wt. each, were selected from in-house cultures and individually rinsed with DI water and gently blotted dry with filter paper. Worms were subsequently weighed to the nearest milligram and randomly distributed to test vessels, with one worm per vessel. Test vessels were incubated under the same conditions used for the *E. andrei* toxicity test (i.e., at room temperature of approximately 20ºC under a light regime of 16:8 h light:dark and light intensity of approximately 150 lux). DI water was added weekly to each test vessel to maintain soil moisture content.

Subsamples of whole earthworms were collected from independent site soil replicates at days 0, 0.4, 1, 2, 4, 7, 10, 14, 17, and 21 of the test for determination of total metal concentrations. Subsamples of whole earthworms were collected from AS replicates at the beginning of the test and on day 21 for determination of background tissue concentrations. Earthworms collected for tissue residue analyses were individually rinsed with DI water and weighed to determine wet weight change throughout the test. After weighing, worms were allowed to purge their gut contents for 24 h by placing them into glass dishes with moistened filter paper. Worms were rinsed again, transferred to pre-weighed liquid scintillation (LS) vials,

and placed into a drying oven at 90ºC. After drying for 24 h, the dry weight of each worm was obtained and samples were stored at 4ºC until analysis.

Subsamples of site soils and AS were collected in triplicate on day 0 and 21 of the bioaccumulation test for total metal analyses.

2.2.6. Toxicity tests

Chronic reproduction tests with earthworms (*E. andrei*) and collembola (*F. candida*), and definitive plant tests with northern wheatgrass (*E. lanceolatus*) and alfalfa (*M. sativa* L.) were performed using the soils and following Environment Canada test methods [98-100]. Earthworms and collembola organisms were obtained from cultures maintained at Stantec Consulting Ltd., Guelph, Ontario, Canada. Northern wheatgrass and alfalfa seeds were purchased from Hannah Seeds, Lacombe, Alberta, Canada and William Dam Seeds Ltd., Dundas, Ontario, Canada, respectively. For the earthworm test, two reproductively mature earthworms were allocated to each 500-mL glass test vessel containing site soil or AS. Adults were removed after 35 days of exposure and adult survival was measured. Test vessels were incubated for another 28 days after which the number of progeny were counted and individual progeny wet and dry mass were measured. For the collembola test, ten age-synchronised organisms were added to each 125-mL glass test vessel containing site soil or AS. Twenty-eight days later, the number of surviving adults and progeny produced were counted. For each plant test, ten (alfalfa) or five (northern wheatgrass) seeds were planted in each 1-L plastic test vessel and incubated under controlled conditions. Following 21 days of exposure, percent emergence, shoot and root length, and shoot and root dry mass were measured. In addition, alfalfa shoot and root tissue metal residues grown in soils A, C and E, as well as AS, were determined from three randomly selected replicates per soil following the 21 day exposure period. For the earthworm and collembola tests, ten and five replicates were used, respectively,

for all site soils and AS. Five replicates of each site soil and AS were used for plant species, with one exception. Due to limited soil quantities, only four replicates were used for testing plant species with soil E.

2.2.7. Tissue and soil analyses

Soil and extracts from the chemical extractions were analysed for total metal content by ALS Laboratory Group in Waterloo, Ontario, Canada. Soil samples were digested with repeated additions of HNO_3 and H_2O_2 and analysed using inductively coupled plasma mass spectrometry (ICP-MS). $CaCl_2$, cyclodextrin, and SEG fluid extracts were prepared for analysis by appropriate additions of HNO_3, and analysed using ICP-MS.

Worm and plant tissue analyses, as well as soil samples from the earthworm bioaccumulation tests, were analysed for metals by Dr. William Hendershot at McGill University in Montreal, Quebec, Canada. Metal concentrations in soil were extracted using a Milestone microwave digestor and 70% HNO_3 (trace-metal grade) and measured using ICP-MS with microwave extraction. Metal concentrations in tissue were extracted using 70% HNO_3 (trace-metal grade) digestions in open tube vessels, and measured with ICP-MS.

Each laboratory performed standard quality assurance procedures including the concurrent analyses of blank, duplicate, and reference samples.

2.2.8. Statistical analyses

Descriptive statistics (i.e., mean and standard deviation) were determined for all endpoints. All statistical analyses were performed using Systat (Version 12). Analysis of variance procedures (ANOVAs) were applied to the toxicity data and weight change throughout the bioaccumulation test with *E. andrei* to determine significant differences among soils. If ANOVA tests indicated a significant different among soils, pairwise comparison Fisher's LSD tests were applied to the data to discriminate differences between means. The assumptions of

normality, homogeneity of variance, and independence were examined; if data failed the Levene's test for equality of variances or the Shapiro-Wilk test for normality, non-parametric Wilcoxon signed rank tests were performed with toxicity data. 2-sided Dunnett's tests were performed with plant tissue concentrations.

Earthworm tissue concentrations measured throughout the bioaccumulation test were corrected for background concentrations (determined from unexposed worm tissues). Determination of kinetic rate constants and kinetic day 21 worm tissue concentrations were performed using the following one-compartment kinetic bioaccumulation equation (1):

$$C_a = (k_u/k_e)*C_s(1-e^{-ket})$$

where C_a is the concentration of metal in worms, and is dependent on the uptake and elimination rate constants, k_u and k_e, respectively, and the total soil concentration, C_s.

The relationship between each measure of bioavailability and observed toxicity was examined using linear regression. *E. andrei* tissue concentrations were only compared with invertebrate biological responses. Univariate linear regression analysis was performed using Systat to compare the average extractable concentration (and average day 21 earthworm tissue concentration) of a single metal (on a mg/kg basis) to toxicity data. Multivariate regression analysis was performed to examine the combined relationships between "bioavailable concentrations" of Cu, Pb, and Zn with each toxicity dataset. Where appropriate, toxicity data were log-transformed for the regression analyses. The results of the univariate and multivariate regressions were compared—multivariate regressions were preferred to assess the strength of each measure of bioavailability. However, where bioavailable concentrations of Cu, Pb, and Zn significantly autocorrelated, the results of the univariate regressions were used to assess the strength of the bioavailability measure. Significance levels of each relationship were determined from the associate *p* value of the slope(s).

2.3. Results

2.3.1. Site soil characteristics

Characteristics of the site soils and AS are presented in Table 2.1. Characteristics such as pH and texture were similar among the site soils whereas other characteristics such as carbon content, OM content, and CEC varied among soils.

2.3.2. Soil extractions

2.3.2.1 Quality control results

Concentrations of Cu and Pb were below detection limits (0.01 mg/L for both) in blank $CaCl_2$, cyclodextrin, and SEG extraction solutions. Zn was detected in two $CaCl_2$ blank solution samples at a concentration of 0.08 mg/L but was not detected in cyclodextrin or SEG blank samples. This was not expected to influence the results of the $CaCl_2$ extractions and soil extracts were not corrected for background values, consistent with Houba *et al*. [118]. Percent recoveries of spiked solutions were between 80-120%.

2.3.2.2 Site soil results

The soil concentrations of Cu, Pb, and Zn as determined by the various extraction procedures are listed in Table 2.2. Total concentrations for each metal generally increased from soil A to E. Concentrations of Cu and Zn in soil E exceeded Ontario site condition standards (225 mg/kg and 600 mg/kg respectively), and concentrations of Pb in soils B-E exceeded the provincial standard of 200 mg/kg [28]. $CaCl_2$-extractable metals did not increase from soils A-E. $CaCl_2$ extractions were not efficient at extracting Cu from the site soils, as Cu was only detected in extracts of soil E, the soil with the highest total Cu concentration. Cyclodextrin extracted more Cu and Pb than $CaCl_2$, and more Pb than the SEG test, although the variation among

cyclodextrin replicates was very high. Extractable amounts of Zn using the SEG test were similar to those extracted with the $CaCl_2$ method but higher than the cyclodextrin extractions. Of the three chemical measures of bioavailability, the SEG test extracted the most Cu from the soils.

Extractable metal concentrations in the contaminated site soils (B, C, D, and E) were lowest in soil C using the $CaCl_2$ and cyclodextrin extractions despite having higher total concentrations of some metals than soils B and D. SEG-extractable Pb and Zn were lowest for soil C, although mobilised Cu was second highest of the site soils. It is evident that the estimated bioavailable fraction of each metal, as well as, the relative estimated bioavailability of a particular metal in one soil compared with another, differs with extraction technique. For example, extractable Zn was similar between soils A and C using the $CaCl_2$ extraction, but the SEG test results indicate that extractable Zn was lower in soil C compared to soil A. Comparing the estimated bioavailable fractions with the results of the toxicity tests should show which methodology is the best predictor of adverse effects.

Table 2.1. Physico-chemical characteristics of the test soils (AS = artificial soil, A = reference soil, B-E = contaminated site soils, *n* =1).

Characteristic	Soil					
	AS	A	B	C	D	E
pH	6.85	7.65	7.87	7.81	7.92	7.92
CEC (cmol+/kg)	-	32.4	14	23.3	19.9	23.3
Total C (% dry)	6.92	6.01	3.2	6.37	5.33	6.65
Organic C (% dry)	6.79	4.27	1.76	3.35	2.82	4.57
OM (% dry)	6.4	8.6	3	3.1	4.2	4.9
P (mg/kg dry wt.)	17	6	26	14	15	32
N (%dry)	0.14	0.21	0.12	0.15	0.15	0.17
Gravel (% wet wt.)	0.0	0.2	20.5	21.1	19.5	18.1
Clay (% wet wt.)	13.9	7.6	6.2	6.1	6.6	10
Silt (% wet wt.)	8.4	5.5	16.1	20	24.2	23.3
Sand (% wet wt.)	77.7	86.9	77.7	73.9	69.2	66.7
Texture	Fine Sandy Loam	Loamy Sand	Gravelly Loamy Fine Sand	Gravelly Coarse Sandy Loam	Sandy Loam	Sandy Loam

pH determined using 0.01 M $CaCl_2$ method [119]
CEC: Cation exchange capacity

Table 2.2. Metal concentrations in the soil, mean ± standard deviation (A = reference soil, B-E = contaminated site soils, *n* =3).

Metal Concentrations (mg/kg dry wt.)					
Soil	Element	Total	$CaCl_2$-extractable fraction	Cyclodextrin-extractable concentration	SEG-extractable concentration
A	Cu	17.9 ± 0.4	ND	0.21 ± 0.00	1.6 ± 0.12
	Pb	89.0 ± 2.0	0.35 ± 0.21	0.11 ± 0.18	0.05 ± 0.05
	Zn	55.7 ± 4.5	3.3 ± 0.2	0.74 ± 0.00	5.0 ± 0.4
B	Cu	56.4 ± 1.4	ND	0.56 ± 0.32	5.6 ± 0.1
	Pb	454.6 ± 78.2	0.40 ± 0.14	2.7 ± 2.4	0.09 ± 0.02
	Zn	227.3 ± 3.7	3.7 ± 0.1	2.3 ± 1.5	4.1 ± 0.2
C	Cu	213.2 ± 42.8	ND	0.38 ± 0.30	18 ± 2
	Pb	661.9 ± 153.3	0.30 ± 0.14	0.42 ± 0.55	0.04 ± 0.04
	Zn	464.4 ± 40.0	3.4 ± 0.1	0.42 ± 0.72	3.6 ± 0.2
D	Cu	176.6 ± 14.2	ND	2.1 ± 1.4	16 ± 1
	Pb	1457.5 ± 59.7	1.5 ± 0.2	14 ± 14	0.31 ± 0.02
	Zn	437.8 ± 19.9	3.7 ± 0.2	3.9 ± 3.4	3.9 ± 0.1
E	Cu	728.1 ± 37.3	0.13 ± 0.06	2.1 ± 1.2	31 ± 1
	Pb	1854 ± 673	1.2 ± 0.2	6.3 ± 5.6	0.14 ± 0.06
	Zn	1100 ± 61	6.6 ± 0.2	3.7 ± 3.0	4.7 ± 0.2

ND: Not detected

2.3.3. Bioaccumulation of metals by plants

Metal analyses results for alfalfa plants used in the toxicity testing are indicated in Fig. 2.1 and Fig. 2.2. Plants grown in soils C and E accumulated more of some metals in their roots and shoots than those grown in the reference soil A. Cu accumulation in plants was similar among the three site soils and AS. Surprisingly, plants grown in AS accumulated more Zn in their tissue than those grown in the reference soil and soil C.

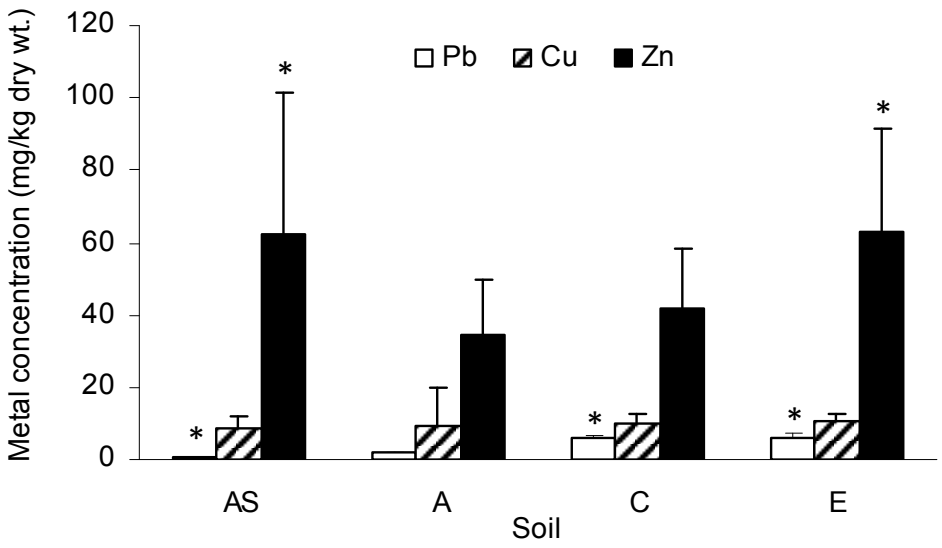

Fig. 2.1. Metal concentrations in alfalfa shoots following 21 days of exposure to artificial soil (AS), a field reference soil (A), and two contaminated site soils (C and E), *n* =3. Error bars indicate one standard deviation of the mean. Significant differences between the AS, soil C, and soil E with the reference soil A are indicated with an asterisk (*) as determined through Dunnett's 2-sided tests ($p < 0.05$).

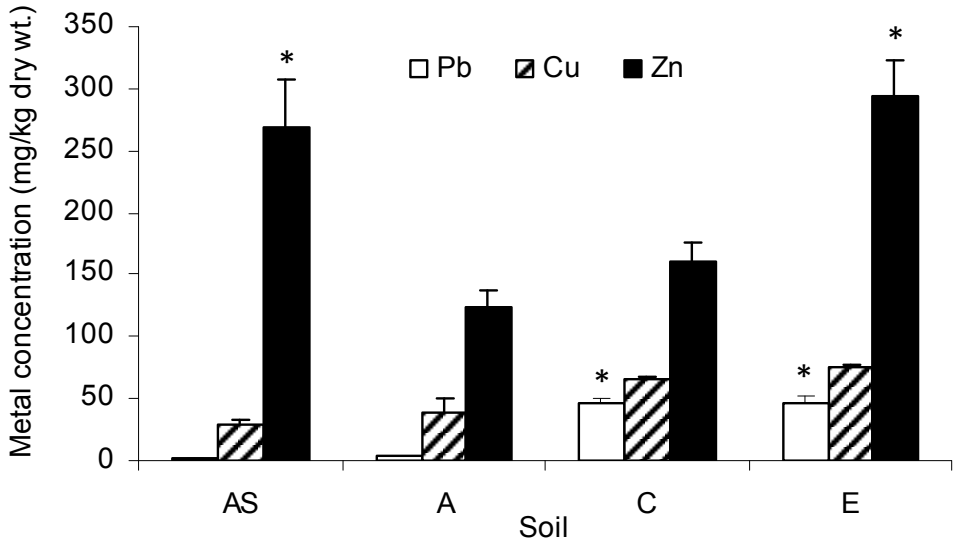

Fig. 2.2. Metal concentrations in alfalfa roots following 21 days of exposure to artificial soil (AS), a field reference soil (A), and two contaminated site soils (C and E), n =3. Error bars indicate one standard deviation of the mean. Significant differences between the AS, soil C, and soil E with the reference soil A are indicated with an asterisk (*) as determined through Dunnett's 2-sided tests ($p < 0.05$).

2.3.4. Bioaccumulation of metals by worms

Earthworm kinetic constants were not calculated for any of the metals investigated since none of the appropriate models [95] could be fit to the data. In general internal metal concentrations either remained constant throughout the uptake phase, or metals accumulated slowly throughout an initial ten day period and reached an apparent steady state by day 21. Detailed accumulation data for each metal are provided in Appendix B. Average internal *E. andrei* concentrations of each metal at day 21 are presented in Table 2.3. Internal Cu and Zn concentrations after 21 days of exposure were lowest in worms exposed to the reference soil, and highest in worms exposed to soil E. Internal Pb concentrations were highly variable, indicating that Pb uptake may be determined on an individual organism basis. The lowest and highest internal Pb concentrations were observed in *E. andrei* exposed to soils A and D, respectively.

Throughout the bioaccumulation test, no overt signs of toxicity were observed when sampling earthworms, and no avoidance behaviour was observed. Weights of earthworms sampled on day 21 increased from day 0 weights in all soils (Fig. 2.3); there were no statistical differences among worms exposed to different soils although weight gain appeared reduced in soils C, D, and E.

Table 2.3. *Eisenia andrei* tissue metal concentrations following 21-days exposure to test soils, mean ± standard deviation (A = reference soil, B-E = contaminated site soils, *n* =3).

	Day 21 tissue concentration (mg/kg dry wt.)		
Soil	Element		
	Cu	Pb	Zn
A	11.3 ± 1.1	5.0 ± 1.1	88.5 ± 15.3
B	19.0 ± 3.9	37.4 ± 22.6	107.2 ± 4.4
C	30.0 ± 7.7	14.4 ± 18.6	95.8 ± 18.4
D	40.7 ± 18.3	120.4 ± 120.8	116.3 ± 37.4
E	44.4 ± 18.0	48.3 ± 33.1	135.2 ± 52.5

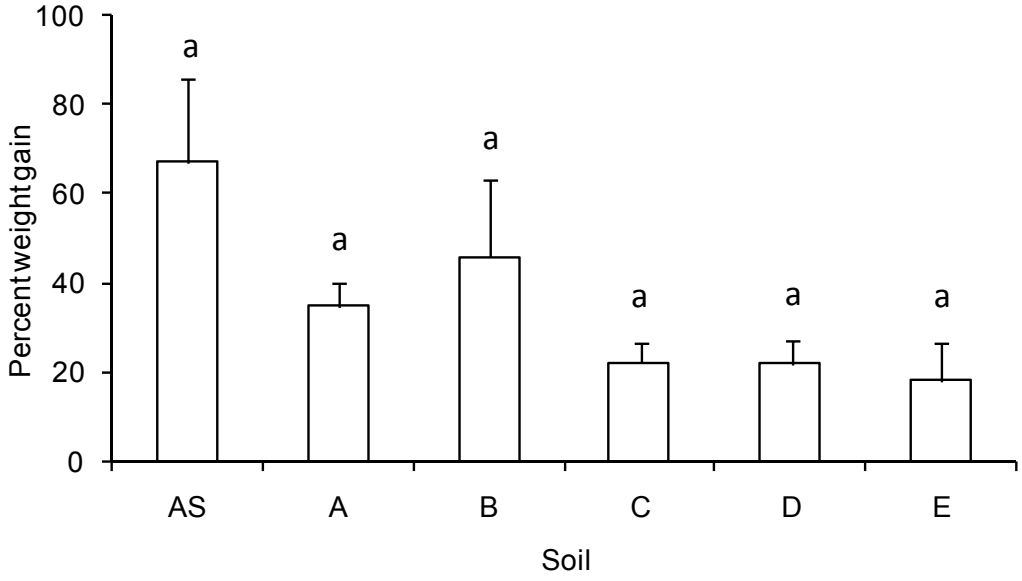

Fig. 2.3. *Eisenia andrei* wet weight gain following 21 days of exposure to artificial soil (AS), reference site soil (A), and contaminated site soils (B, C, D, E). Error bars indicate one standard deviation of the mean. Columns with the same letter indicate no significant differences at $p > 0.05$; $n = 3$.

2.3.5. Toxicity tests

All organisms generally performed best in the uncontaminated AS and site reference soil A. Alfalfa and northern wheatgrass seedling emergence was at least 90% in all soils (data not shown). Although shorter in contaminated soils, alfalfa root lengths were similar among soils B to E despite a wide range of total metal concentrations in soil (Fig. 2.4). Shoot and root mass were inhibited most in soil C for both alfalfa (Fig. 2.5) and northern wheatgrass (Fig. 2.7) despite soil C being only moderately contaminated relative to the other site soils, but an adverse effect on northern wheatgrass root length following exposure to soil C was not observed (Fig. 2.6). In soils B to E, a clear pattern of increased adverse effects to either alfalfa or northern wheatgrass with increased total metal concentration is absent; in fact, soil E, which exceeded provincial standards for all three metals, does not appear to be the most toxic soil to plants.

In general, soil E had no more of an effect than soils C or D to invertebrates, although it had a more significant effect on *F. candida* progeny production than the next highest contaminated soil D (Fig. 2.8, Fig. 2.9, and Fig. 2.10). In general, invertebrate effects data were highly variable with large standard deviations. Thus, determining a significant difference between site soils was problematic. Also, the lack of progeny produced in some replicates, specifically for soils C and E, decreased the *n* (i.e., the number of test units per soil) used in the statistical analyses of *E. andrei* progeny wet and dry weights. Adult *E. andrei* survival following 35 days of exposure was at least 95% in all soils (data not shown).

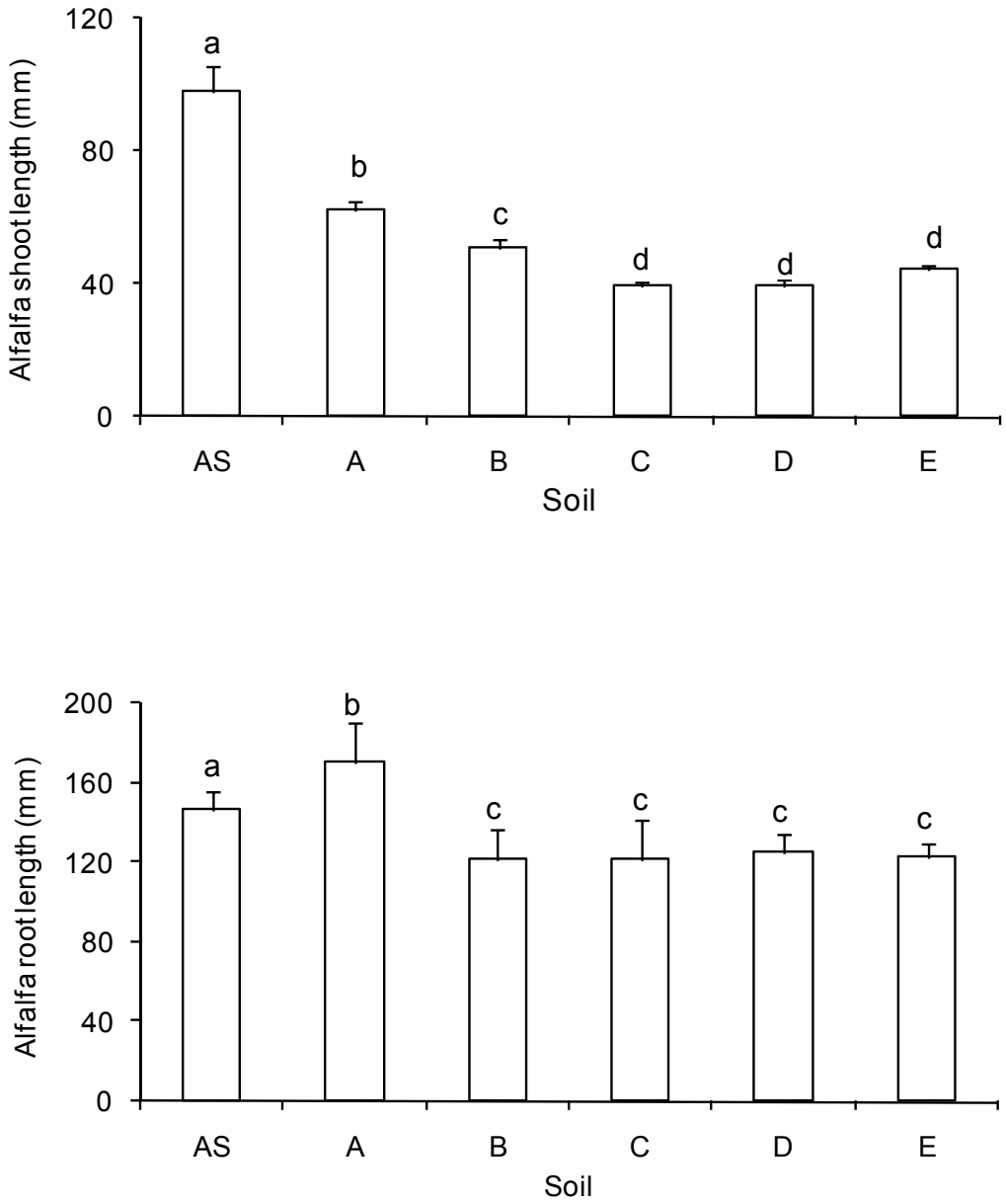

Fig. 2.4. Average alfalfa shoot and root length following 21-days exposure to site soils and AS (AS = artificial soil, A = reference site soil, B-E = contaminated site soils). Error bars indicate one standard deviation of the mean. Columns with the same letter indicate no significant differences at $p > 0.05$; n =4 or 5, depending on the soil.

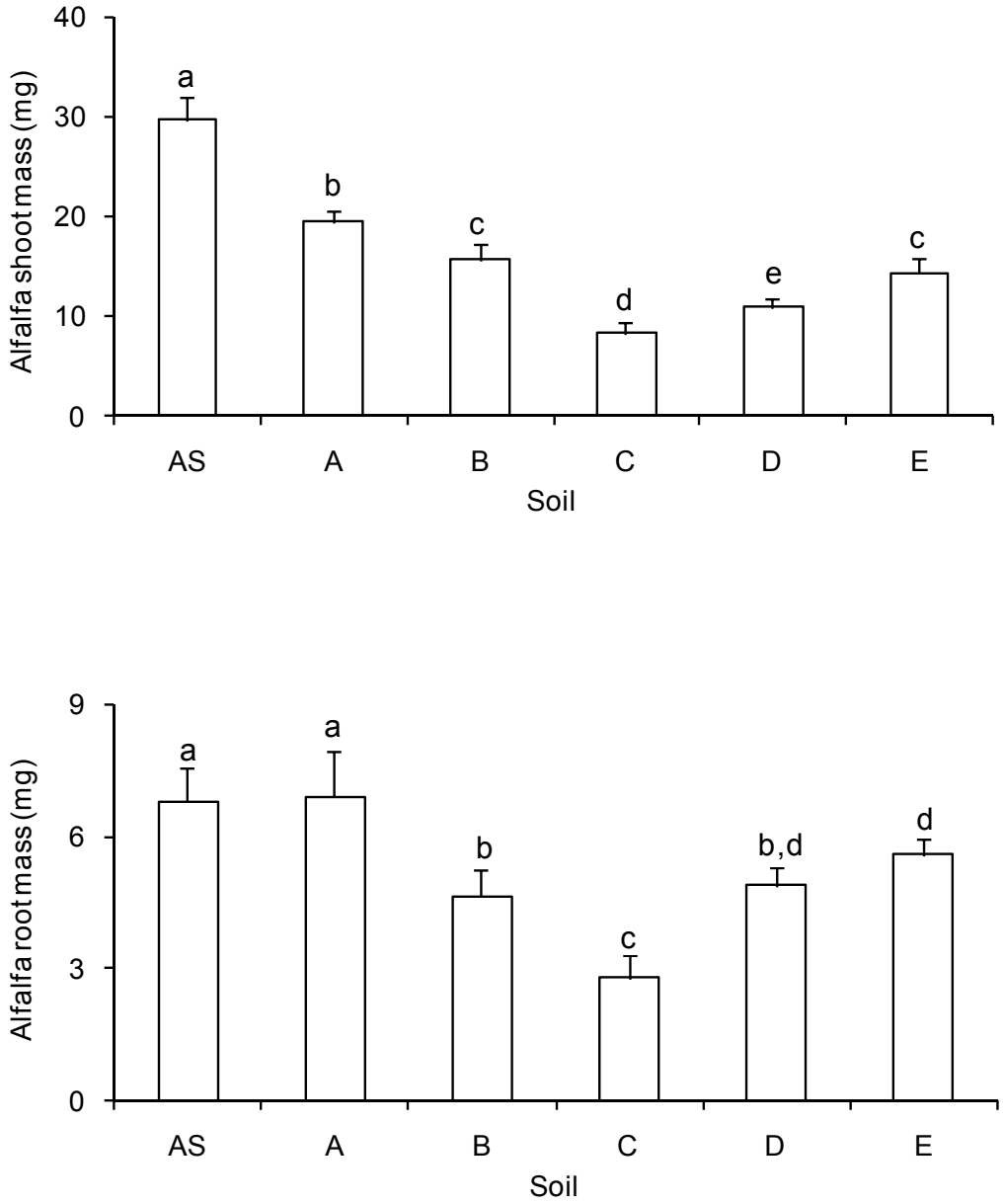

Fig. 2.5. Average alfalfa shoot and root dry mass following 21-days exposure to site soils and AS (AS = artificial soil, A = reference site soil, B-E = contaminated site soils). Error bars indicate one standard deviation of the mean. Columns with the same letter indicate no significant differences at $p > 0.05$; n =4 or 5, depending on the soil.

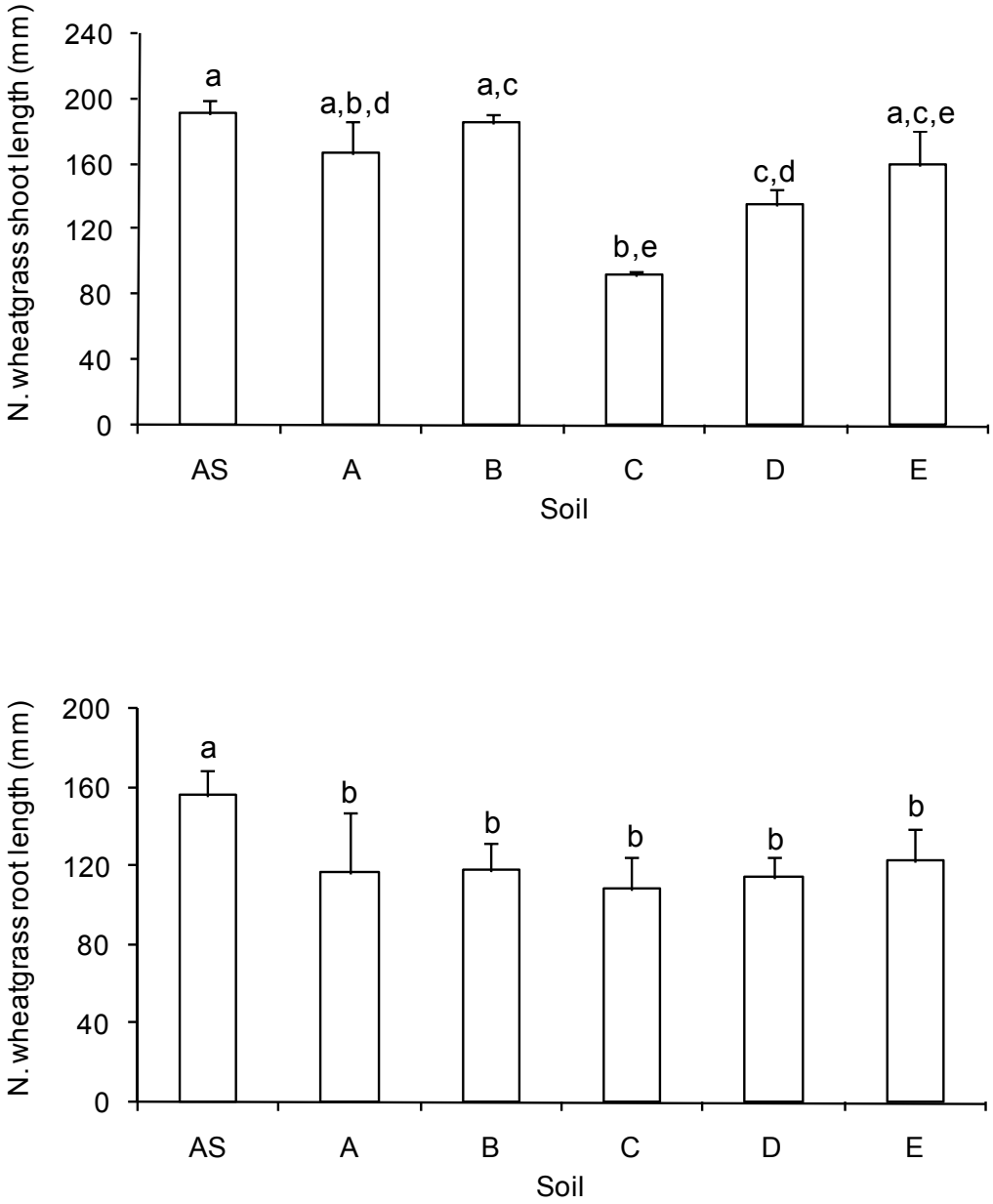

Fig. 2.6. Average northern wheatgrass shoot and root length following 21-days exposure to site soils and AS (AS = artificial soil, A = reference site soil, B-E = contaminated site soils). Error bars indicate one standard deviation of the mean. Columns with the same letter indicate no significant differences at $p > 0.05$; n =4 to 5, depending on the soil.

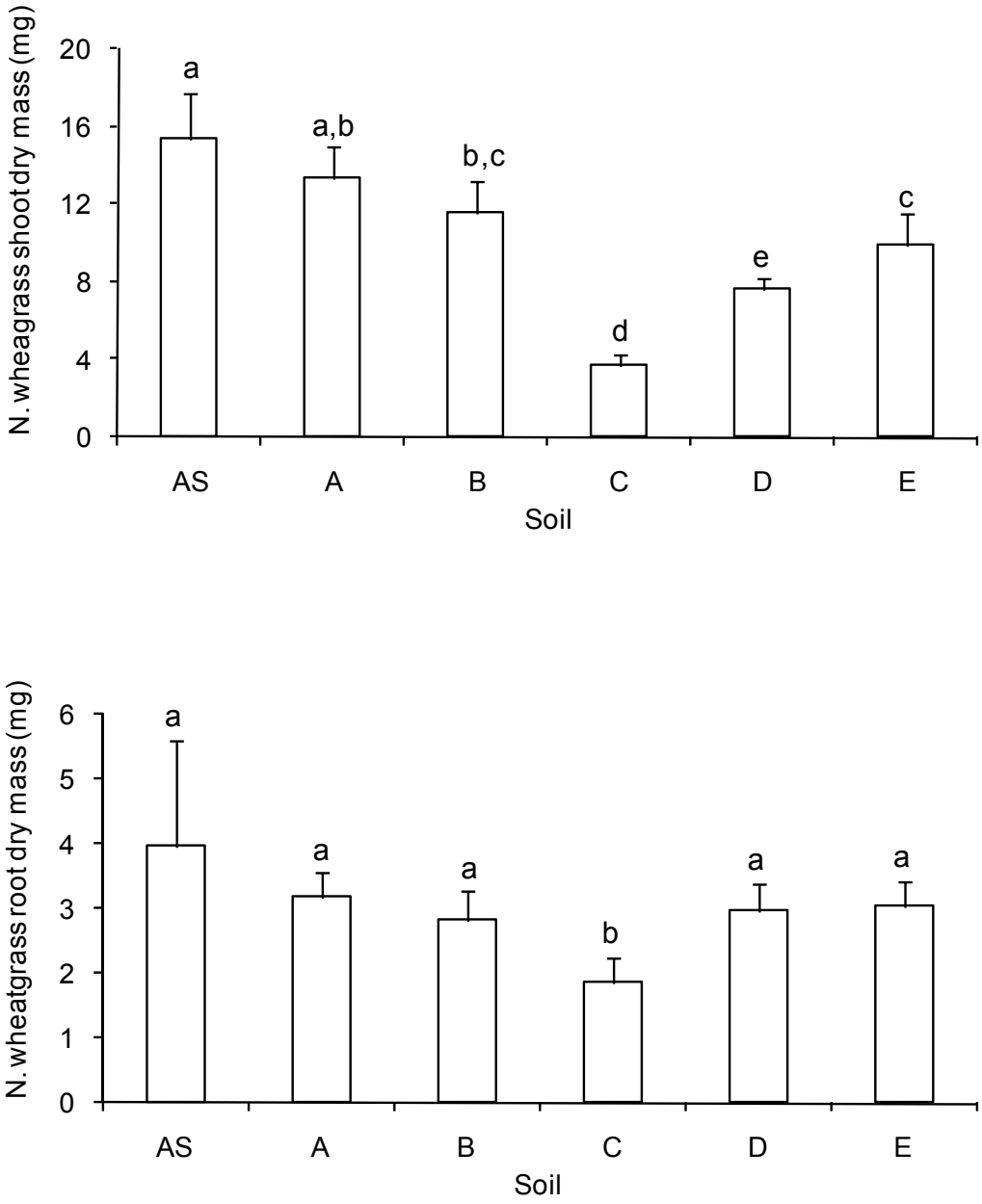

Fig. 2.7. Average northern wheatgrass shoot and root dry mass following 21-days exposure to site soils and AS (AS = artificial soil, A = reference site soil, B-E = contaminated site soils). Error bars indicate one standard deviation of the mean. Columns with the same letter indicate no significant differences at $p > 0.05$; n =4 to 5, depending on the soil.

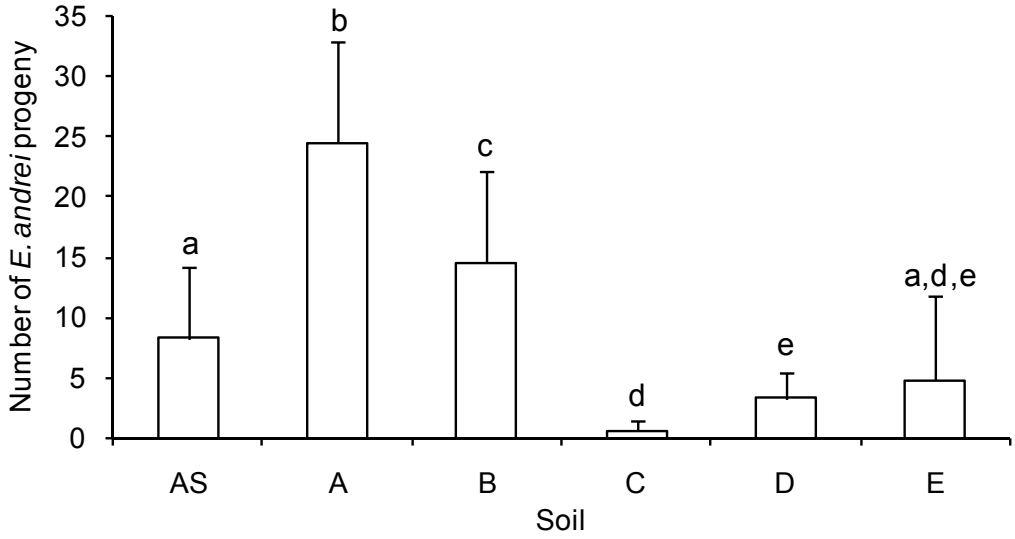

Fig. 2.8. Mean number of *Eisenia andrei* progeny produced following 63-days exposure to site soils and AS (AS = artificial soil, A = reference site soil, B-E = contaminated site soils). Error bars indicate one standard deviation of the mean. Columns with the same letter indicate no significant differences at $p > 0.05$; $n = 10$.

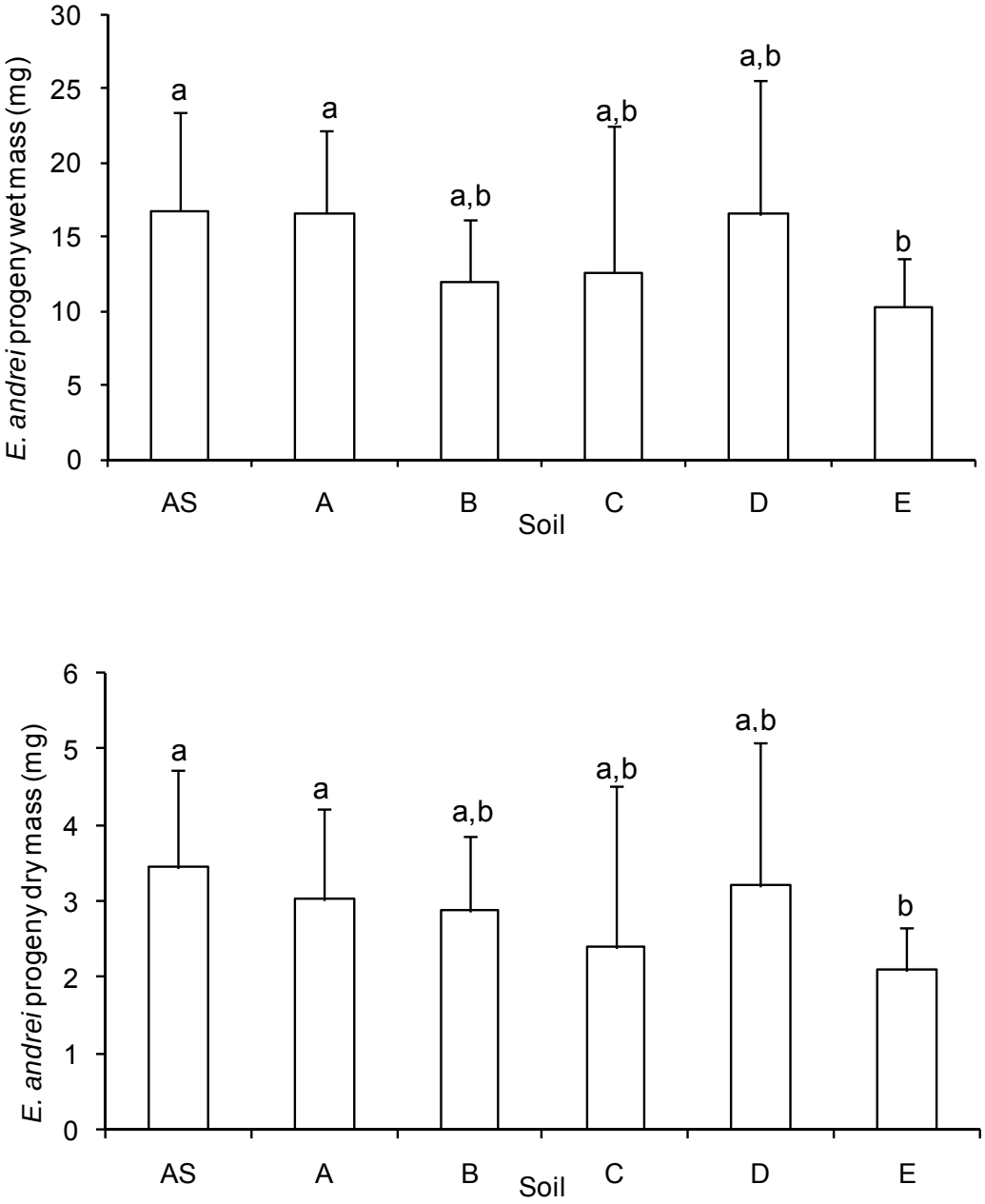

Fig. 2.9. Mean mass of *Eisenia andrei* progeny following 63-days exposure to site soils and AS (AS = artificial soil, A = reference site soil, B-E = contaminated site soils). Error bars indicate one standard deviation of the mean. Bars with the same letter indicate no significant differences at $p > 0.05$; n was as follows: AS, 10; A, 9; B, 10; C, 4; D, 9; and E, 6.

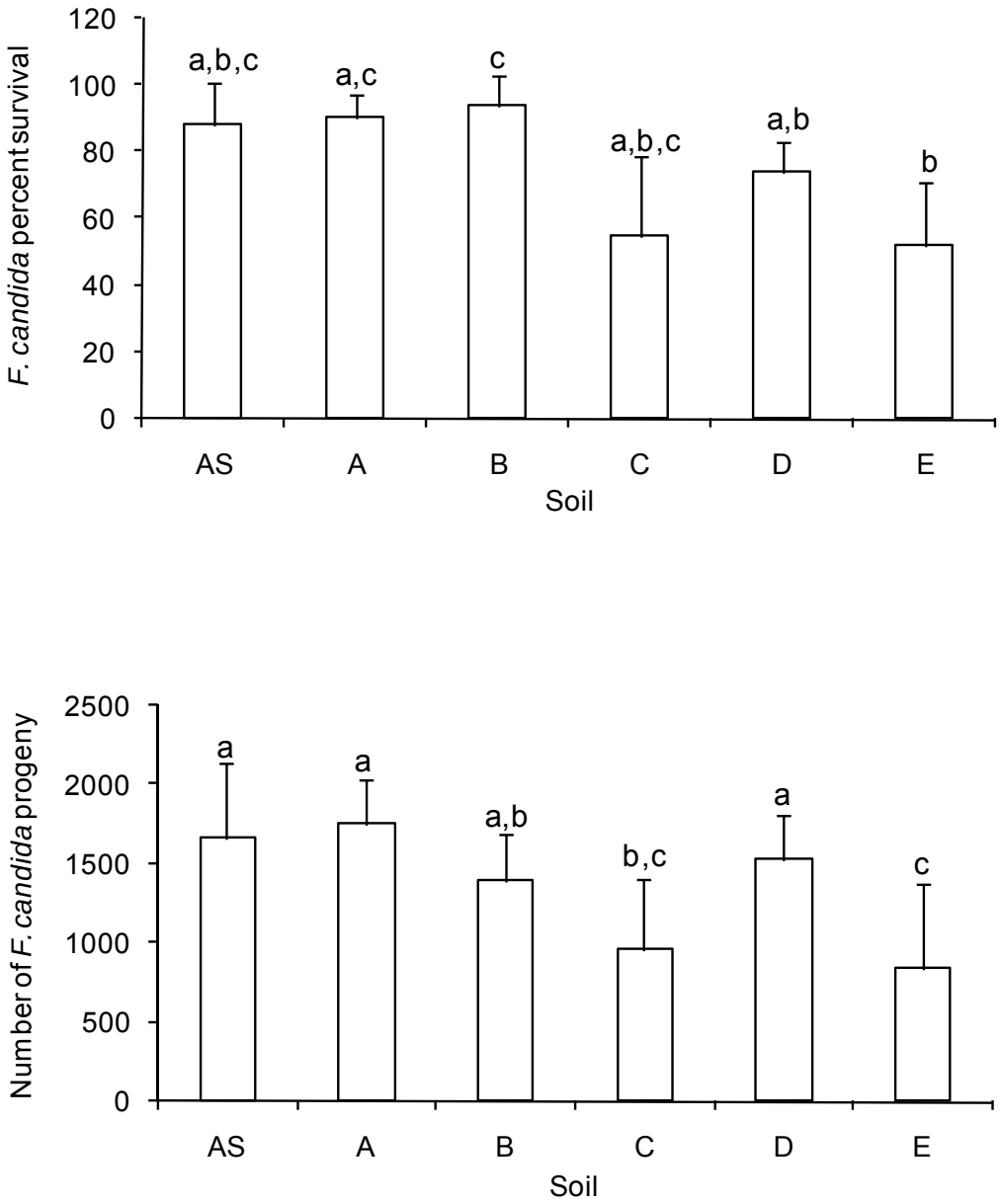

Fig. 2.10. Effects on survival and reproduction of *Folsomia candida* following 28-days exposure to site soils and AS (AS = artificial soil, A = reference site soil, B-E = contaminated site soils). Error bars indicate one standard deviation of the mean. Bars with the same letter indicate no significant differences at $p > 0.05$; $n = 5$.

2.3.6. Comparison of results

Total and $CaCl_2$-extractable concentrations of metals autocorrelated to a high degree (i.e., correlation coefficients between the *x* variables were less than -0.8); therefore, the estimates obtained through multivariate regression were not used. High autocorrelation or multicollinearity indicates extreme dependence of the *x* variables [120]. Instead, the results of univariate regressions were used for comparison purposes. Cyclodextrin-extractable, SEG-extractable, and *E. andrei* tissue concentrations were compared with the toxicity test results using multivariate regressions. Adjusted coefficients of determination and standard coefficients for biological endpoints with a significant difference among soils are listed in Table 2.4.

The correlations between effects data generated for a single species or among species were not determined. However, it is important to note that the relative correlations between a measure of bioavailability and two biological endpoints can be used to gain some insight into the potential correlations between those two biological endpoints. For example, the SEG test correlated well with both *E. andrei* progeny production (adjusted $r^2 = 0.587$) and alfalfa shoot length (adjusted $r^2 = 0.929$). The SEG-extractable Cu concentration was indicative of adverse effects in both cases. This indicates that *E. andrei* progeny production might be correlated with alfalfa root length. However, these relationships are not discussed further as the correlation of biological endpoints is not the focus of this research.

Table 2.4. Results of univariate and multivariate regressions for the site soils. Concentrations of metals in soils or biological tissues determined using each surrogate measure of bioavailability were compared with effects data. Where concentrations of metals autocorrelate, the adjusted r^2 values of univariate regressions are shown; otherwise, multivariate adjusted r^2 and standard coefficient values are shown.

				Eisenia andrei effects				*Folsomia candida* effects		Northern wheatgrass effects			Alfalfa effects			
Measure	Regression	Value	Metal	No. of progeny	Progeny wet wt.	Progeny dry wt.	Day 21 weight change	Adult survival	No. of progeny	Shoot length	Shoot mass	Root mass	Shoot length	Root length	Shoot mass	Root mass
Total	U	Adjusted r^2	Cu	0.098*	0.039	0.029	0.409**	0.398^	0.282^	0.000	0.000	0.000	0.131	0.063	0.000	0.000
			Pb	0.290^	0.031	0.001	0.423**	0.294^	0.124**	0.000	0.020	0.000	0.405**	0.164*	0.089	0.000
			Zn	0.192^	0.052	0.034	0.437	0.431^	0.314^	0.000	0.025	0.000	0.283*	0.152*	0.049	0.000
$CaCl_2$	U	Adjusted r^2	Cu	0.000	0.024	0.007	0.236*	0.234*	0.185*	0.000	0.000	0.007	0.000	0.000	0.000	0.000
			Pb	0.176**	0.000	0.000	0.224	0.059	0.000	0.000	0.000	0.096	0.176*	0.016	0.000	0.000
			Zn	0.000	0.035	0.005	0.217	0.216*	0.182*	0.011	0.000	0.019	0.000	0.013	0.000	0.000
Cyclodextrin	M	Standard coefficient	Cu	-1.013*	NA	NA	-2.081^	-2.158^	NA	-1.657^	-1.423*	NA	-1.152*	NA	-1.176	NA
			Pb	-0.415	NA	NA	0.212	0.567	NA	-0.726*	-0.611	NA	-0.435	NA	-0.719	NA
			Zn	0.989*	NA	NA	1.421*	1.342**	NA	2.509^	2.008^	NA	1.116*	NA	1.707**	NA
		Adjusted r^2		0.296^	0.000	0.000	0.57**	0.479**	0.200	0.663^	0.374*	0.179	0.342*	0.000	0.296*	NA
SEG	M	Standard coefficient	Cu	-0.448^	NA	NA	-0.734**	-0.780^	-0.679^	-0.296	-0.385^	-0.171	-0.507^	-0.364*	-0.376^	-0.217*
			Pb	-0.020	NA	NA	-0.077	0.188	0.354*	0.220	0.166	0.505^	-0.181**	-0.012	0.019	0.324^
			Zn	0.53^	NA	NA	-0.019	0.125	0.184	0.644^	0.776^	0.678^	0.631^	0.524**	0.806^	0.884^
		Adjusted r^2		0.587^	0.050	0.010	0.45*	0.516^	0.411^	0.442^	0.754^	0.541^	0.929^	0.418^	0.857^	0.822^
Eisenia andrei tissue concentration	M	Standard coefficient	Cu	-1.392	NA	NA	-1.196*	-1.375	-0.814*	ND	ND	ND	ND	ND	ND	ND
			Pb	0.127	NA	NA	0.280	0.609	0.654**	ND	ND	ND	ND	ND	ND	ND
			Zn	0.740	NA	NA	0.340	0.396	-0.075	ND	ND	ND	ND	ND	ND	ND
		Adjusted r^2		0.561^	0.021	0.006	0.515*	0.585^	0.361**	ND	ND	ND	ND	ND	ND	ND

*: $p < 0.05$

**: $p < 0.01$

^: $p < 0.005$

M: Multivariate

U: Univariate

NA: Not applicable because multivariate regression was not significant ($p > 0.05$)

ND: Not determined

2.3.6.1 Comparison of measures of bioavailability with effects to Eisenia andrei

The $CaCl_2$ extraction was the worst predictor of adverse effects to *E. andrei* (Table 2.4). Of all of the measures, the SEG test and *E. andrei* tissue concentrations correlated best (adjusted $r^2 > 0.5$ and $p < 0.005$) with the mean number of progeny produced by *E. andrei*, and the SEG test was a marginally better predictor based on the adjusted coefficient of determination (r^2) value alone (0.587 versus 0.561). Furthermore, no single metal in *E. andrei* tissue was significantly correlated with progeny production ($p > 0.05$ for all metals), whereas the SEG-extractable Cu concentration was positively correlated with the number of progeny ($p < 0.005$) and Zn was negatively correlated with effects ($p < 0.005$). *E. andrei* tissue Cu concentrations and SEG-extractable Cu concentrations, along with total metal and cyclodextrin-extractable Cu concentrations, were predictive of *E. andrei* weight change after 21-days of exposure during the bioaccumulation experiment (Table 2.4). For the latter extraction, Zn was positively correlated with *E. andrei* weight increase ($p < 0.05$). Cyclodextrin-extractable concentrations and, to a lesser extent, *E. andrei* tissue metal concentrations were highly variable (Table 2.2 and Table 2.3), and there is limited confidence in the correlations between these measures of bioavailability and observed effects. If an extraction technique is highly variable and imprecise, it cannot reliably be used to estimate bioavailability.

No significant correlations were noted between any measures of bioavailability and effects on progeny weights (Table 2.4). This could be, in part, due to the influence of the more toxic soils in which only zero to three progeny were counted in a single replicate. It is likely that the few progeny weighed may have been more tolerant of metal concentrations in those soils and biased the results.

2.3.6.2 Comparison of measures of bioavailability with effects to Folsomia candida

While some metal concentrations in some tests were significantly correlated with adverse effects (Table 2.4), the adjusted coefficients of determination were less than ideal (i.e., below 0.5). The SEG test was the only extraction method that had a good correlation with *F. candida* adult survival (adjusted $r^2 > 0.5$, $p < 0.05$). In this case, effects were correlated with SEG-extractable Cu. Multivariate regression of *E. andrei* tissue concentrations for Cu, Pb, and Zn correlated best with adult survival, but no single metal was predictive of survival (Table 2.4).

The SEG test correlated best with the mean number of *F. candida* progeny produced (Table 2.4). However, no measure of bioavailability, including the SEG test, was a good predictor (adjusted $r^2 < 0.5$).

2.3.6.3 Comparison of measures of bioavailability with effects to northern wheatgrass

Neither total nor $CaCl_2$-extractable metal concentrations were predictive of adverse effects to northern wheatgrass (Table 2.4). No measure of bioavailability correlated with northern wheatgrass root lengths. Cyclodextrin predicted shoot length effects well (adjusted $r^2 = 0.663$, $p < 0.005$), with effects attributed to Cu concentrations ($p < 0.005$) and to a lesser extent, Pb concentrations ($p < 0.05$); Zn negatively correlated with adverse effects ($p < 0.005$). As discussed earlier, the confidence in cyclodextrin-extractable metal concentrations is low due to their high variability. Therefore, the SEG test was considered to be the best predictor of shoot length (Table 2.4), although the correlation was less than ideal (adjusted $r^2 < 0.5$).

The SEG test was the best predictor of effects on northern wheatgrass shoot and root mass (adjusted $r^2 = 0.754$ and 0.541, respectively, and $p < 0.005$; Table 2.4). However, effects were only correlated with a particular metal (i.e., Cu) in one instance (i.e., shoot mass, $p < 0.005$). SEG-extractable Zn was negatively correlated with adverse effects to northern

wheatgrass ($p < 0.005$), and Pb was negatively correlated with adverse effects on root mass ($p < 0.005$).

2.3.6.4 Comparison of measures of bioavailability with effects to alfalfa

SEG-extractable metals, particularly Cu, were the best predictors of adverse effects to alfalfa (Table 2.4). Multivariate regression of SEG-extractable metals with alfalfa shoot length, shoot mass, and root mass fit very well, with regression coefficients greater than 0.8 ($p < 0.005$). Cu was positively correlated, and Zn was negatively correlated, with adverse effects for all endpoints. Pb had a relatively small contribution to alfalfa fitness (Table 2.4). No other measure of bioavailability adequately predicted adverse effects.

2.3.7. Summary of results

Tissue concentrations of Cu were similar in alfalfa grown in three of the site soils, and tissue concentrations of Pb and Zn increased with increasing total metal concentration in the site soils. Tissue concentrations of Cu and Pb in *E. andrei* exposed to site soils were soil-dependent, and tissue concentrations of Zn in *E. andrei* were marginally soil-dependent. Plant species performed worst in soil C. Soil E, which had the highest total metal concentrations, had no greater of an effect to invertebrates and plants (and in some cases, soil E had less of an effect) than soil C. Through comparison of the results of the measures of bioavailability with the results of the toxicity tests, no surrogate measure of bioavailability reliably predicted effects on all endpoints for all organisms. However, the SEG test was, in most cases, a better predictor of adverse effects that other surrogate measures of bioavailability and total concentrations of metals in soil.

2.4. Discussion

Ecological receptors such as earthworms have been observed inhabiting field soils with extremely high total metal concentrations. Their presence was accounted for by adaptation processes and low metal bioavailability [23]. The present results lend further credence to the idea that total concentrations of metals in soil are not useful for predicting toxicity to ecological receptors. Pore water is thought to be the main source of exposure through direct contact with metals in soil to invertebrates and plants [15, 16, 46], or at least an uptake route which is related to the soil porewater concentration [46, 47]. Therefore, biological and chemical measures of bioavailability are ideal alternatives for expressing metal concentrations in soil. Chemical extractions are useful for predicting bioavailability to ecological receptors. However, chemical extractions are only useful when the contaminant of interest (at environmentally relevant concentrations) can be detected in extracts from soils, and when the extraction and analytical technique are precise.

Pb detection issues were encountered by Spurgeon *et al*. [40] and Grelle and Decamps [72] using low concentrations (0.01 M) of $CaCl_2$; however, the higher concentration of $CaCl_2$ used for this study was expected to eliminate such issues. Pb was present at concentrations above the detection limit in all of the $CaCl_2$ extracts, but Cu was not detected the extracts for all soils except the most highly contaminated soil E. Since $CaCl_2$ was the “weakest” extractant used herein, the majority of Cu in these soils was probably not easily mobilised. Soil properties have a profound influence on Cu partitioning in soils [35]; Cu binds strongly to soil OM [39, 73] and bioavailability may not be predicted by pore water concentrations of Cu [7].

The cyclodextrin extract concentrations were highly variable for all contaminants in all soils. At first, this does not appear to be attributed to soil heterogeneity, as the other chemical measures were relatively precise. However, perhaps percent organic carbon was heterogeneous among samples. This could have a profound influence on the cyclodextrin

extraction results, more so than the other extractions, because it is expected to extract organometallic forms of metals. Cyclodextrin was the most efficient extractant of Pb relative to the $CaCl_2$ extraction and SEG test. This could indicate that a large portion of total Pb content in the soils is bound to organic material. It is unclear as to why the cyclodextrin extract concentrations were so variable but, as such, its use to predict the bioavailable concentration of metals in these soils is unreliable. Further investigations and research are required to determine whether this high variability in extract concentrations is common using other soils.

Tissue concentrations of Cu were similar among alfalfa plants grown in the three site soils and AS. As well, the tissue concentrations of Zn did not appear to be dependent on total metal concentrations, consistent with other reports (e.g., [60]). Although Cu and Zn may accumulate in some plants from some soils, internal levels of these metals are likely regulated [51, 73]. Lock and Janssen [7] determined that plant tissue concentrations of Cu were regulated only below a threshold porewater concentration which corresponded to a 0.01 M $CaCl_2$-extractable concentration of approximately 30-50 mg Cu/kg soil. The $CaCl_2$-extractable concentrations of Cu in this Chapter were several orders of magnitude below this threshold, which probably explains why Cu did not accumulate in alfalfa. Pb was the only metal that appeared to bioaccumulate from these soils, which was attributed to its non-essentiality. Unfortunately, there were not sufficient data to reliably perform regression analyses to correlate plant tissue concentrations with plant toxicity. Future experiments should investigate this biological measure of bioavailability further using similar regression procedures.

Uptake and elimination of Cu and Zn are controlled to a certain extent by earthworms; this accounts for the inconsistent uptake patterns observed in *E. andrei* in this Chapter. Unlike non-essential metals such as Cd, there is typically not a clear phase of rapid uptake observed in kinetic bioaccumulation studies [17, 18, 50, 83]. Instead, concentrations may remain initially unchanged, rapidly increase and subsequently decrease to initial levels, or follow no clear

pattern at all. It is possible that uptake of essential metals reaches some internal threshold after which excretion mechanisms are activated and the metals are eliminated or the organism dies [6, 23]. Other metals, such as Pb, are stored internally by sequestration within inorganic matrices or binding to organic ligands [17]. One would expect that concentrations of Pb would continue to accumulate over time. This was not observed in this study, as well as others (e.g., [50, 104]). It is possible that the internal Pb storage complexes are eliminated from *E. andrei*. However, Spurgeon and Hopkin [6] observed a non-linear increase in *E. fetida* concentrations of Pb throughout 42 days of exposure. Comparing bioaccumulation using soils from different sites can be problematic as co-contaminants and mixtures can affect bioaccumulation kinetics [17, 42].

Ecotoxicity tests provided extremely useful data regarding site soil toxicity. Soil C could be marginally the most toxic to almost all species tested, a result that would not have been inferred based on analyses of total metal concentrations alone. It is probable that soil characteristics directly affected the fitness of the battery of test organisms. Before commencing the toxicity tests, nutrient levels (e.g., phosphorus and nitrogen) were determined in the site soils (Table 2.1), and based on these determinations, supplementation with plant fertilisers was deemed unnecessary. Nevertheless, nutrient availability could have had a direct or indirect effect on an organism's fitness, particularly for plant species. Progeny production for sexually mature earthworms is influenced by the amount of OM content in soils; although capable of surviving, adult earthworms rarely reproduce in soils when the OM content is less than 4%. The low OM content may have been a contributing factor to the responses of organisms exposed to soils C (and B). Invertebrate responses were highly variable in each soil, including AS.

The usefulness of the *E. andrei* progeny wet and dry weight endpoints for these soils are questionable; for some soils (e.g., soils C and E), no progeny were produced in many of the replicates. This reduced the number of *n* in the statistical analyses of progeny weight data (i.e.,

test vessels with no progeny produced were not considered in the statistical analyses of progeny weights). In the more "toxic" soils, where juveniles were present in a replicate at the end of the exposure period, the replicate often contained only one to five juveniles. If, say, only one juvenile survived in a particular test vessel, it is possible that this organism either had increased tolerance to metal contamination and is less susceptible to adverse effects on growth or simply benefited from more food because of the lack of competition. Therefore, when this individual organism is used to represent all *E. andrei* exposed to that particular soil, the results may be skewed. The comparison of the surrogate measures of bioavailability with *E. andrei* toxicity data lends credibility to this assertion because no measure correlated with progeny weights.

The presence of mixtures of contaminants in soil makes it difficult to determine which specific contaminant is most responsible for toxic responses, particularly if contaminant concentrations autocorrelate. Most likely, Cu, Pb, and Zn are all contributing to the responses observed in this study. Mixture effects can be additive, less-than-additive, or synergistic, and it has been suggested that metal mixture toxicity to soil-dwelling organisms is additive or less-than-additive [18]. For these results, most of the multivariate comparisons between measures of bioavailability and effects data indicated that a single metal (i.e., Cu) positively correlated with the observed adverse effects, and that Zn often negatively correlated with adverse effects. Total and $CaCl_2$-extractable concentrations were compared with effects data using univariate regressions, which were required because of autocorrelation. Some uncertainty exists when dealing with contaminant mixtures; however, it is rare to encounter contaminated sites in ecological risk assessment that have only one contaminant issue.

Neither the measures of bioavailability nor total metal concentrations were predictive of effects on *E. andrei* progeny weights. Disregarding these data based on the previous discussion, the SEG test and internal *E. andrei* concentrations were the best predictors of

adverse effects to *E. andrei*. The relatively quicker SEG method, as well as its relatively high precision, makes it an ideal method for predicting toxicity and bioavailability to *E. andrei* in these soils. The $CaCl_2$ extraction, which is correlated with porewater concentrations of metals [7] was not predictive of adverse effects to *E. andrei*. The SEG formulation likely solubilises metals that weak extractants (i.e., $CaCl_2$) cannot, although the predictive capability of the $CaCl_2$ formulation may have been compromised by detection limit issues.

The SEG was designed to estimate the bioaccessible fraction of metals from soil. The primary route of contaminant uptake is dictated by several factors including soil characteristics, contamination type, chemical speciation, and earthworm species. Cd and Zn concentrations in *L. terrestris* with their mouths sealed (i.e., exposure was only through the dermal route) were 83% and 79%, respectively, of concentrations detected in *L. terrestris* able to ingest contaminated soil (i.e., exposure was through both dermal and oral uptake) [104]. Saxe *et al*. [26] estimated that 96% of total Cd and Cu uptake and 82% of total Zn uptake occurs through the dermal route using modeling procedures. However, emphasis on the role of the gut in metal uptake by earthworms has been reported in other studies [16, 84, 105]. Ultimately, the relative importance of dermal and ingestion exposure routes is dependent on the bioavailable and bioaccessible metal concentrations [39]. It is possible that metals strongly bound to OM in soil and on which earthworms selectively feed are taken up in the gut and this uptake would not be accounted for in chemical extractions designed to estimate porewater concentrations [104]. Therefore, the good correlation between the SEG test and *E. andrei* effects may indicate that oral exposure is an important route of uptake of Cu by earthworms.

Although the SEG test was developed based on earthworm gut composition, it was also consistently one of the best predictors of adverse effects to *F. candida*, northern wheatgrass, and alfalfa. Effects were generally positively correlated with bioavailable Cu. Like *E. andrei*, its correlation with *F. candida* toxicity could indicate that a large proportion of Cu exposure in these

soils is via the oral route. Previous studies reported that $CaCl_2$-extractable metals correlate with invertebrate toxicity [7, 40, 46] and plant toxicity [38]. The detection limit issues for Cu extracted with 0.5 M $CaCl_2$ may account for the utter lack of any correlation between this extraction and biological endpoints.

The correlation between SEG-extractable metals (particularly Cu) and adverse effects to plants is somewhat surprising given the SEG's intended purpose. However, the enzymatic composition of the SEG formulation may simulate microbial processes in the rhizosphere and release organically-bound metals. While it is generally believed that plant exposure to some metals is via porewater uptake, it has been suggested that organically-bound Cu is more phytoavailable than Cu associated with inorganic precipitates and the residual fraction [67]. Alternatively, the presence of enzymes may have increased the ionic strength of the SEG solution, and the good correlation between extractable metals and adverse effects to plants is attributable to competition with soil absorption sites similar to a weak salt extraction.

The SEG test correlated best with adverse effects for two invertebrate species and two plant species in comparison with other measures of bioavailability (and total metal concentrations in soil), despite the observation that typically, while one extraction technique may be a good predictor of bioavailability in one species, it may be a poor predictor with another [8]. It is likely that the correlation of the other measures of bioavailability with biological endpoints was confounded somewhat by responses observed in organisms exposed to soil C. Extractable concentrations of metals in this soil were typically lower than those for soils D and E using most measures of bioavailability (i.e., $CaCl_2$, cyclodextrin, and *E. andrei* concentrations) and total metal analysis. It is possible that the toxicity of soil C was not attributed solely to the bioavailable metal portion and instead partially attributable to soil characteristics. Besides affecting bioavailability, soil characteristics such as pH and OM content can be stress factors themselves and affect the fitness of the organism and thereby its sensitivity to the toxicant [121].

OM was relatively low in soil C. Alternatively, perhaps soil characteristics in soils D and E were somehow mitigating toxicity of the bioavailable metals to invertebrate and plant organisms. However, the SEG extraction did indicate that Cu was second-most bioavailable in soil C relative to the other soils. Further comparisons using other field soils with different physico-chemical characteristics are required to better understand these relationships.

2.5. Conclusions

It is clear that neither total metal concentrations nor chemical extraction techniques fully predicted the bioavailability, and hence toxicity, of metals in these soils to the battery of test species. Earthworm tissue concentrations provided a relatively good indication of adverse effects to invertebrates, but effects were not correlated with concentrations of a metal or metals, and tissue concentrations of Pb were highly variable. No surrogate measure of bioavailability reliably predicted effects on all endpoints for all organisms, but some, particularly the SEG test, were better predictors than total metal concentrations. The novel SEG test shows the most promise for predicting toxicity of metals in soil, although further research is needed. Ultimately, Tier 1 ecological benchmarks could be adjusted using a surrogate measure of bioavailability such as the SEG test when it has been further validated.

2.6. Summary

Bioavailability is a major factor affecting toxicity of metals in soil to ecological receptors. Metal concentrations in soil are often compared to Tier 1 ecological benchmarks, which are based on total concentrations in soil. Often, the total concentration is not correlated with toxicity. No standardised method exists for determining the bioavailability of contaminants in soil to ecological receptors. Several surrogate measures of bioavailability were compared to the results of a battery of toxicity tests using Cu, Pb, and Zn-contaminated soils collected from a

former industrial area. A $CaCl_2$ extraction, cyclodextrin extraction, SEG test, and earthworm bioaccumulation test were performed using the soils. Extractable metals using the $CaCl_2$ solution were not correlated with any biological responses of *E. andrei*, *F. candida*, northern wheatgrass, or alfalfa. Concentrations of metals in the cyclodextrin extracts were highly variable and were not adequate for prediction of effects. The SEG test correlated best with most of the biological endpoints. Bioavailable Cu was correlated with adverse effects to invertebrates and plants using the SEG test. *E. andrei* tissue concentrations were variable but were predictive of adverse effects to invertebrates. Overall, the SEG test was more predictive of effects than the other surrogate measures of bioavailability, including total concentrations in soil, likely due to enzymatic activity and increased ionic strength of the solution. Further validation is required before this test is routinely used to estimate metal bioavailability.

CHAPTER 3

ESTIMATING BIOAVAILABILITY OF COPPER AND ZINC IN MINING SOILS

3.1. Introduction

Human reliance on metals for electronic and electrical devices, steel, automotive parts, glasses, paints, and so on, necessitate the excavation of large amounts of ore from the earth [67]. This typically results in metal contamination surrounding excavation areas, partially because non-recovered metals are released as wastes [67]. Over time, a significant amount of waste rock and tailings may accumulate in soils. This material is dispersed through physical processes such as wind and rain [89], and results in contamination of the environment. Although various remediation techniques have been proposed (e.g., [89]), often the contaminated material (i.e., soil) is simply removed from the site and deposited as land fill. However, this process can be very expensive, and since metals are neither created nor destroyed, it does not remove the contamination, it merely relocates it [89].

Elevated concentrations of metals, as determined through extraction techniques used to determine the “total” metal content, are often encountered at current and former mining sites. This poses a problem to regulators and environmental assessors as the total concentrations of metals in soils at mining sites often exceed jurisdictional guidelines (i.e., Tier 1 ecological benchmarks). Exceedance of jurisdictional guidelines does not necessarily indicate that metal concentrations are hazardous to ecological receptors. Contaminants that have been in soil for a prolonged period can become recalcitrant over time due to various physico-chemical and

biological processes (e.g., ageing, weathering, sequestration, adsorption, degradation etc.) [5, 6, 8, 9], decreasing the bioavailability of the contaminant. Bioavailability is the amount of a contaminant that is taken up by an organism from an external environmental media. Bioavailability directly influences toxicity.

When total concentrations of metals in soil exceed Tier 1 ecological benchmarks, site-specific tests are sometimes used to assess the toxicity of those soils to ecological receptors as part of the Tier 2 process. Site-specific toxicity testing has been used at mining sites to determine if soils with elevated total metal concentrations impaired habitat quality for earthworms, and/or impaired growth of cress (*Lepidium sativum* L.) [74]. Site-specific ecotoxicity tests are used to assess the quality of contaminated soils at mining sites [46, 74]. Ecotoxicity testing is an indirect measure of bioavailability, and the effects on the measured responses (i.e., reproduction) are attributable, in part, to exposure to the bioavailable portion of metal in the soil. If an alternate laboratory test was available that could accurately predict metal bioavailability, it could in theory be used as a surrogate for ecotoxicity testing. For such a test to be a viable alternative, it should be extensively validated with biological endpoints, as well as, offer time, resource, and cost saving benefits in comparison to ecotoxicity testing.

As mentioned in Chapter 1, two approaches can be taken to estimate bioavailability: chemical and biological measures. The former includes chemical extractions, biomimetic devices, bioaccessibility testing, and modeling, and the latter includes bioaccumulation and ecotoxicity tests. Each of these tools has advantages and limitations and has proven useful when applied within a specific research context. Bioaccumulation testing and measuring tissue residues in biota has been suggested as a good indicator of toxicity (e.g., [15, 50, 80]), and chemical extractions are indicative of bioaccumulation of some metals in terrestrial organisms (e.g., [39, 48]). To develop a practical test for metal bioavailability, a chemical method must be validated with the bioavailable metal pool in the soil or some biological measure [15, 54].

However, there are no standardised methods for measuring bioavailability and no one method can be recommended over another because there are insufficient comparative or applied data.

In Chapter 2, results of a $CaCl_2$ extraction, cyclodextrin extraction, SEG test, and an earthworm bioaccumulation test were compared to the results of a battery of toxicity tests to determine which surrogate measure of bioavailability correlated best with effects of Cu, Pb, and Zn contaminated soils to ecological receptors. Of the four surrogate measures, the novel SEG test best predicted bioavailability and adverse effects to ecological organisms. Metal toxicity to invertebrates and plants was attributed to the bioavailable fraction of Cu.

The objectives of the research presented within this Chapter were to compare surrogate measures of bioavailability to a battery of toxicity tests using organic soils from a former mining site contaminated with Cu and Zn. In addition to the tests used in Chapter 2, red clover tissue concentrations following 14 days of exposure to contaminated soils were compared to a battery of toxicity tests using invertebrates and plants. The soils used herein differ substantially from those used in Chapter 2 both in contamination type, contaminant concentration, and physico-chemical characteristics. The correlations between measures of bioavailability and biological endpoints will be used to indicate whether the SEG test, as well as, other measures of bioavailability, are predictive of adverse effects of highly organic soils contaminated by metal mining activities.

3.2. Methods

3.2.1. Site characteristics and soil collection

Soils were collected from a former Cu and Zn mining site in Quebec, Canada. Mining activities ceased in early 2004 after more than 20 years of operation. Samples were collected from three general areas on the Site: areas impacted by wet and dry deposition of airborne metal-contaminated dust (C3-1, C3-2, and C3-3), areas impacted by the overflow of acidic,

metal contaminated water from a former tailings pond (B3-1 and B3-2), and an area unimpacted by mining activities (C5-1, located upgradient of the prevalent wind direction). All areas were natural mature forest environments with highly organic (peaty) soils. At each sampling location, approximately 20 kg of soil were collected from the top 10 cm after removal of plant material, as only the surficial soil layer was expected to be contaminated in some areas. All site soils were air-dried, thoroughly homogenised, and sieved to ≤6 mm. Total concentrations of Cu and Zn in the contaminated soils exceeded jurisdictional guidelines.

AS was formulated based on Environment Canada test methods as described in Chapter 2. The maximum WHC and physico-chemical characteristics of the site soils and AS were measured. WHC capacity was determined by saturating 50 g of dry soil with DI water, allowing the wetted soil to sit in a covered glass funnel, and weighing the wet soil after three hours of draining. Physico-chemical analyses were performed by the Soil Nutrient Laboratory at the University of Guelph, Guelph, Ontario, Canada.

3.2.2. Soil manipulation

The pH of impacted soils ranged from 3.80 to 4.76, as measured in a 2:1 (v:v) DI water to soil slurry after collection. *E. andrei*, a common earthworm species used in ecotoxicity and bioaccumulation testing, can be tested in many field soils but reproduction is hindered in acidic soils [41]. Also, most of the plant species recommended by Environment Canada [98] do not grow well in acidic soils. Powdered calcium carbonate ($CaCO_3$) has been used to raise the pH of acidic soils [40] and is used in the formulation of AS. Therefore, a fraction of each contaminated soil was amended with powdered $CaCO_3$ to increase the soil pH to within 0.5 units of the reference soil, C5-1. *F. candida* was recently recommended as a potential collembolan test species for the testing of soil from Canadian boreal forests and northern lands [102], and is tolerant to acidic soils. Therefore, soils used in the ecotoxicity testing with

F. candida were not manipulated to increase pH. The low pH of soils was not expected to impact adult *E. andrei* survival, so amended soils were not used in the *E. andrei* 21-day bioaccumulation test. Unamended soils were also used for the $CaCl_2$, cyclodextrin, and SEG extractions. In summary, amended soils were used only for the *E. andrei* and plant ecotoxicity tests.

3.2.3. $CaCl_2$, cyclodextrin, and SEG extractions

0.5 M $CaCl_2$, 0.0035 M cyclodextrin, and SEG extractions were performed using unamended site soils as described in Chapter 2 with the following exceptions: 1) Out of necessity because of the very high WHC of the site soils, the liquid to soil ratios used in the extractions were double those described in Chapter 2 so that the solutions added were not completely absorbed by the organic soils and supernatant could still be collected following extraction. For example, whereas only 5 g of dry soil were added to each test vessel, the volume of 0.5 M $CaCl_2$ solution added remained the same (i.e., 100 mL); and 2) $CaCl_2$ samples were not centrifuged before filtration and analyses. This was an unnecessary step because preliminary testing indicated that a large fraction of OM remained in the supernatant regardless of centrifugation speed and time.

3.2.4. Earthworm bioaccumulation test

Generally, the 21-day earthworm bioaccumulation test was performed using procedures as described in Chapter 2 following the draft OECD guideline [95]. *E. andrei* were exposed individually to unamended site soils for a period of 21 days. Subsamples of whole earthworms were collected from independent site soil replicates at days 1, 2, 4, 7, 14, and 21 of the test for determination of total metal concentrations. Three earthworms were collected from each site soil at each sampling event. The number of replicates was determined before commencing the test based on the number of sampling events and number of replicates sampled each sampling

event. In addition, three additional test units per soil were allocated as “back-ups” in the event of earthworm mortality or sample loss.

3.2.5. Toxicity tests

Chronic reproduction tests with earthworms (*E. andrei*, 63-day duration) and collembola (*F. candida*, 28-day duration), and definitive plant tests with northern wheatgrass (*E. lanceolatus,* 21-day duration) and red clover (*T. pratense*, 14-day duration) were performed using either the amended (*E. andrei*, northern wheatgrass, and red clover) or unamended (*F. candida*) soils following Environment Canada test methods [98-100]. Organisms were procured from the same sources identified in Chapter 2; red clover seeds were purchased from William Dam Seeds Ltd., Dundas, Ontario, Canada.

Effects on adult survival (invertebrates), progeny production (invertebrates), progeny mass (earthworms), emergence (plants), shoot and root length (plants), and shoot and root dry mass (plants) were measured. In addition, the tissue metal residues of red clover grown in site soils were determined. Three randomly selected replicates of shoot and root tissue were submitted for analyses following the 14 day exposure period. When the root mass produced in individual replicates was too small to accurately quantify metal levels, the samples from each replicate were pooled into one sample (i.e., for plants grown in soils B3-1, B3-2, C3-2, and C3-3).

3.2.6. Tissue and soil analyses

Soil and extracts from the chemical extractions were analysed for total metal content by ALS Laboratory Group in Waterloo, Ontario, Canada. Soil samples were digested with repeated additions of HNO_3 and H_2O_2 and analysed using ICP-MS. Extracts were prepared for analysis by appropriate additions of HNO_3, and analysed using ICP-MS.

Worm and plant tissue analyses, as well as soil samples from the earthworm bioaccumulation tests, were analysed for metals by Dr. William Hendershot at McGill University in Montreal, Quebec, Canada. Metal concentrations in soil were extracted using a Milestone microwave digestor and 70% HNO_3 (trace-metal grade) and measured using ICP-MS with microwave extraction. Metal concentrations in tissue were extracted using 70% HNO_3 (trace-metal grade) digestions in open tube vessels, and measured with ICP-MS.

Quality assurance procedures included the concurrent analyses of blank, duplicate, and reference samples.

3.2.7. Statistical analyses

Descriptive statistics (i.e., mean and standard deviation) were performed on all data. Systat (Version 12) was used for all statistical analyses. ANOVA procedures were applied to the toxicity data and data for mass change of *E. andrei* after 14 days of exposure during the bioaccumulation test to determine significant differences between soils. Day 14 *E. andrei* weights were used because only one earthworm sample was collected from soil B3-1 after 21 days of exposure due to mortality throughout the experiment. If ANOVA tests indicated a significant different among soils, pairwise comparison Fisher's LSD tests were applied to the data to discriminate which means were different. Wilcoxon signed rank tests were performed on data if they failed the Levene's test for equality of variances or the Shapiro-Wilk test for normality.

Earthworm and tissue concentrations were corrected for background concentrations before determination of kinetic uptake parameters. Uptake kinetic day 21 worm tissue concentrations were determined using the kinetic bioaccumulation equation (1) described in Chapter 2.

Each measure of bioavailability was linearly regressed with observed adverse effects. *E. andrei* and red clover tissue concentrations were only regressed with invertebrate and plant biological responses, respectively. Univariate linear regression analysis was used to compare the average extractable concentration (and average day 21 earthworm and red clover tissue concentration) of a single metal (on a mg/kg basis) to effects data. Multivariate regression analysis was performed to examine the combined relationships between bioavailable concentrations of Cu and Zn with each effects dataset. Adverse effects data were log-transformed where appropriate for the regression analyses. Where bioavailable concentrations of Cu and Zn were highly autocorrelated, the results of the univariate regressions were used to assess the strength of the bioavailability measure; otherwise, multivariate regressions were relied upon. The *p* value of each slope was used to determine the significance level of each relationship.

3.3. Results

3.3.1. Site soil characteristics

Physico-chemical characteristics of the site soils and AS are presented in Table 3.1. The pH of the amended soils were within 0.5 units of the reference site soil, C5-1. Since the soils were highly organic, numerous parameters such as OM content and texture could not be reliably determined and are not reported.

Table 3.1. Physico-chemical characteristics of the site soils and artificial soil (AS) (n =1). Natural pH refers to the pH of the soil before amendment with $CaCO_3$, and adjusted pH refers to the pH following amendment.

Characteristic	Soil						
	AS	C5-1	B3-1	B3-2	C3-1	C3-2	C3-3
Natural pH	6.85	6.30	3.80	4.21	3.81	4.76	4.40
Adjusted pH	NA	NA	6.44	6.36	6.34	6.32	6.38
CEC (cmol+/kg)	-	59.8	31.5	50.1	46.2	41.8	44.1
Total C (% dry)	6.92	33.8	40.5	40.4	38.7	35.4	39.8
Organic C (% dry)	6.79	33.6	40.5	40.4	38.7	35.4	39.8
P (mg/kg dry wt.)	17	54	22	12	21	30	55
N (%dry)	0.14	0.99	2.35	2.25	1.06	0.89	1.17

pH determined using a 2:1 (v:v) DI water to soil ratio

CEC: Cation exchange capacity

NA: pH adjustment not necessary

3.3.2. Soil extractions

3.3.2.1 Quality control results

Concentrations of Cu were below detection limits (0.01 mg/L) in all blank extract samples. Zn was detected in $CaCl_2$ and SEG blank solutions at concentrations of 0.12 mg/L and 0.8 mg/L, respectively. Neither Cu nor Zn was detected in DI water used to formulate the extraction solutions. Zn detections in blank samples were not expected to significantly influence the interpretation of the $CaCl_2$ and SEG extraction results and soil extracts were not corrected for background values. Percent recoveries of metals in spiked solutions were between 80-120%.

3.3.2.2 Site soil results

Table 3.2 summarises the mean concentrations of Cu and Zn in soil as determined by the various extraction procedures. Cu concentrations in soil were not correlated with Zn concentrations in soil as determined from total and $CaCl_2$ procedures (regression coefficients of -0.486 and -0.596 respectively). Extractable concentrations of Cu were highly correlated with extractable concentrations of Zn using cyclodextrin and SEG extractions (regression coefficients of -0.999 and -0.939 respectively). Extractable concentrations were lowest in the reference site soil C5-1 for all chemical measures of bioavailability, whereas the relative estimated bioavailability of a particular metal among soils differed with extraction technique.

Table 3.2. Metal concentrations in the soil, mean ± standard deviation (SD, *n* =3).

Soil	Element	Metal Concentrations (mg/kg dry wt.) Total	$CaCl_2$-extractable	Cyclodextrin-extractable	SEG-extractable
C5-1	Cu	77.3 ± 2.0	5.8 ± 0.4	0.67 ± 0.12	2.26 ± 0.17
	Zn	201.3 ± 7.1	122.0 ± 0.4	15.1 ± 3.7	13.9 ± 0.3
B3-1	Cu	2226 ± 13	476.9 ± 1.7	16.0 ± 0.9	29.9 ± 2.4
	Zn	766.8 ± 8.4	659.4 ± 15.8	76.6 ± 4.4	120.1 ± 2.8
B3-2	Cu	1860 ± 71	260.9 ± 0.6	10.6 ± 0.4	15.5 ± 0.7
	Zn	1823 ± 157	1186 ± 17	57.0 ± 7.9	76.5 ± 0.9
C3-1	Cu	1938 ± 58	530.7 ± 49.6	6.67 ± 0.40	17.8 ± 0.5
	Zn	2517 ± 85	1064 ± 173	39.0 ± 7.3	112.6 ± 8.1
C3-2	Cu	949.0 ± 39.0	35.0 ± 0.3	2.98 ± 0.10	6.68 ± 0.84
	Zn	1549 ± 53	461.5 ± 12.1	22.3 ± 1.4	27.8 ± 1.4
C3-3	Cu	1395 ± 27	113.4 ± 0.6	5.72 ± 0.20	7.05 ± 0.55
	Zn	2466 ± 8	888.0 ± 18.8	34.6 ± 8.9	43.1 ± 0.3

3.3.3. Bioaccumulation of metals by plants

Tissue concentrations in the shoots and roots of red clover plants at the end of the 14 day toxicity tests are provided in Table 3.3. The highest Cu and Zn concentrations in shoots were observed in plants grown in soils B3-1 and B3-2, respectively. Surprisingly, Cu concentrations in root tissue were highest in plants grown in the reference site soil. However, plants grown in soil C5-1 had the lowest calcium and Zn concentrations in root tissue; these metals act as competitors for Cu at the root surface and their lower concentrations may account for the increased Cu concentrations in roots. Zn concentrations were highest in the roots of plants grown in soils B3-1, B3-2, and C3-1.

Table 3.3. Red clover shoot and root concentrations of Cu and Zn following 14 days of exposure to site soils, mean ± standard deviation (SD; *n* =3, except where SD is not provided and *n* =1).

Soil	Day 14 tissue concentration (mg/kg dry wt.)			
	Shoot		Root	
	Cu	Zn	Cu	Zn
C5-1	16.8 ± 0.6	55.2 ± 7.4	445 ± 109	175 ± 19
B3-1	25.8 ± 3.0	226.6 ± 12.6	307	2352
B3-2	22.2 ± 0.9	429.3 ± 37.9	251	2383
C3-1	19.7 ± 0.6	311.4 ± 7.0	301 ± 52	2190 ± 204
C3-2	16.1 ± 2.2	279.5 ± 8.6	211	1159
C3-3	19.9 ± 4.9	386.1 ± 15.6	261	1725

3.3.4. Bioaccumulation of metals by worms

Earthworm kinetic constants were not calculated for any of the metals investigated since kinetic models [95] could not be fit to the data. Kinetic constants could not be calculated for *E. andrei* tissue concentrations of Cu and Zn in Chapter 2 either. In general, internal Zn concentrations, corrected for background, remained constant throughout the uptake phase; Cu accumulated slowly throughout an initial seven day period and reached an apparent steady state by day 14. Bioaccumulation data are presented in Appendix B. Internal *E. andrei* concentrations of Cu and Zn on day 21 are presented in Table 3.4. Internal Cu and Zn concentrations were lowest in worms exposed to the reference soil, and highest in worms exposed to soils B3-2 and B3-1, respectively.

Throughout the bioaccumulation test, overt signs of toxicity and avoidance behaviour were observed in some replicates for some of the site soils. Worms were generally located at the bottom of test vessels (i.e., not within the soil) containing soils B3-1 and C3-1 on all sampling events. In addition, dead adults were observed in soil B3-1 after 4 days (n = 2), 7 days (n = 2), and 14 days (n = 1) of exposure and in soil C3-1 following 21 days of exposure (n = 1). No overt signs of toxicity or avoidance behaviour were noted in worms exposed to other soils. Wet weights of earthworms sampled on day 14 increased from day 0 weights in AS, C5-1, C3-2, and C3-3 (Fig. 2.3). Weight loss occurred in worms exposed to soils B3-1, B3-2, and C3-1, and day 14 wet weight change was significantly different than for worms exposed to the reference site soil.

Table 3.4. *Eisenia andrei* tissue metal concentrations following 21-days exposure to site soils, mean ± standard deviation (SD, *n* =3, except for B3-1, where *n* =1).

	Day 21 tissue concentration (mg/kg dry wt.)	
Soil	Element	
	Cu	Zn
C5-1	13.7 ± 1.0	97.4 ± 6.6
B3-1	45.27	152.0
B3-2	58.3 ± 14.1	120.6 ± 18.9
C3-1	23.8 ± 8.5	112.5 ± 2.0
C3-2	42.9 ± 14.8	133.9 ± 29.2
C3-3	48.7 ± 13.6	132.5 ± 34.3

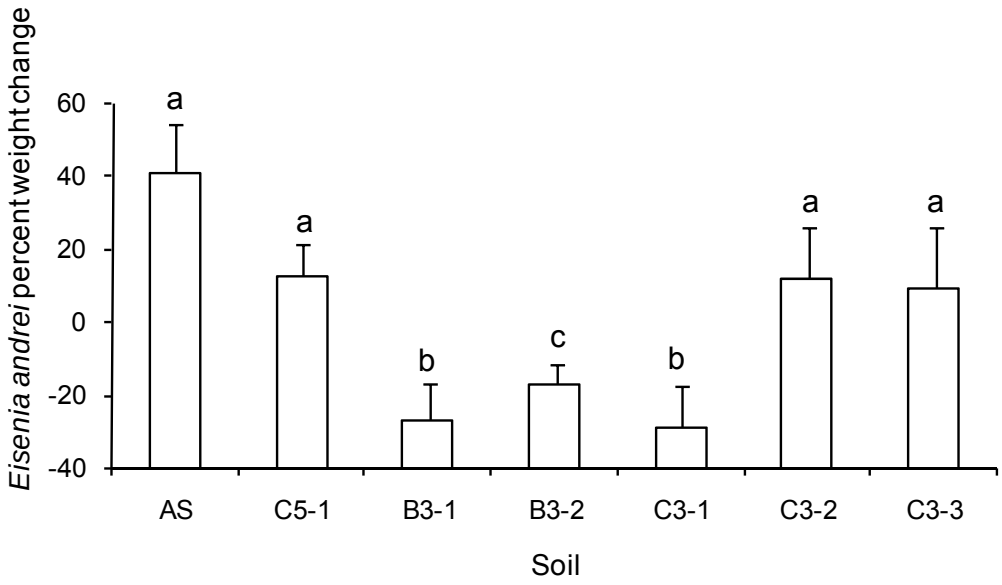

Fig. 3.1. *Eisenia andrei* wet weight change following 14 days of exposure to artificial soil (AS), reference site soil (C5-1), and contaminated site soils. Error bars indicate one standard deviation of the mean. Columns with the same letter indicate no significant differences at $p > 0.05$; $n = 3$.

3.3.5. Toxicity tests

Red clover and northern wheatgrass seedling emergence was at least 85% in all soils and there were no significant differences among soils (data not shown). Otherwise, significant differences in biological responses were observed for plants grown in all contaminated site soils in comparison with the reference site soil. In general, adverse effects were most profound in northern wheatgrass and red clover exposed to soil B3-1, followed by plants exposed to soils B3-2 and C3-1 (Fig. 3.2, Fig. 3.3, Fig. 3.4, and Fig. 3.5).

Adult *E. andrei* survival following 35 days of exposure was 100% in all soils (data not shown). Soils B3-1, B3-2, and C3-1 adversely affected *E. andrei* and progeny were completely absent in all B3-1 replicates, 70% of B3-2 replicates, and 90% of C3-1 replicates (Fig. 3.6). It is expected that the lack of progeny produced in some soils may have influenced the *E. andrei* progeny wet and dry weight data (Fig. 3.7) since the impact that a single worm has on the average weight is much higher for soils B3-2 and C3-1, as discussed in Chapter 2. Effects on *F. candida* adult survival were not significantly different among soils (data not shown). Effects on *F. candida* progeny production were fairly similar among soils, except *F. candida* exposed to soil C3-1 were adversely affected in comparison to the reference site soil (Fig. 3.8).

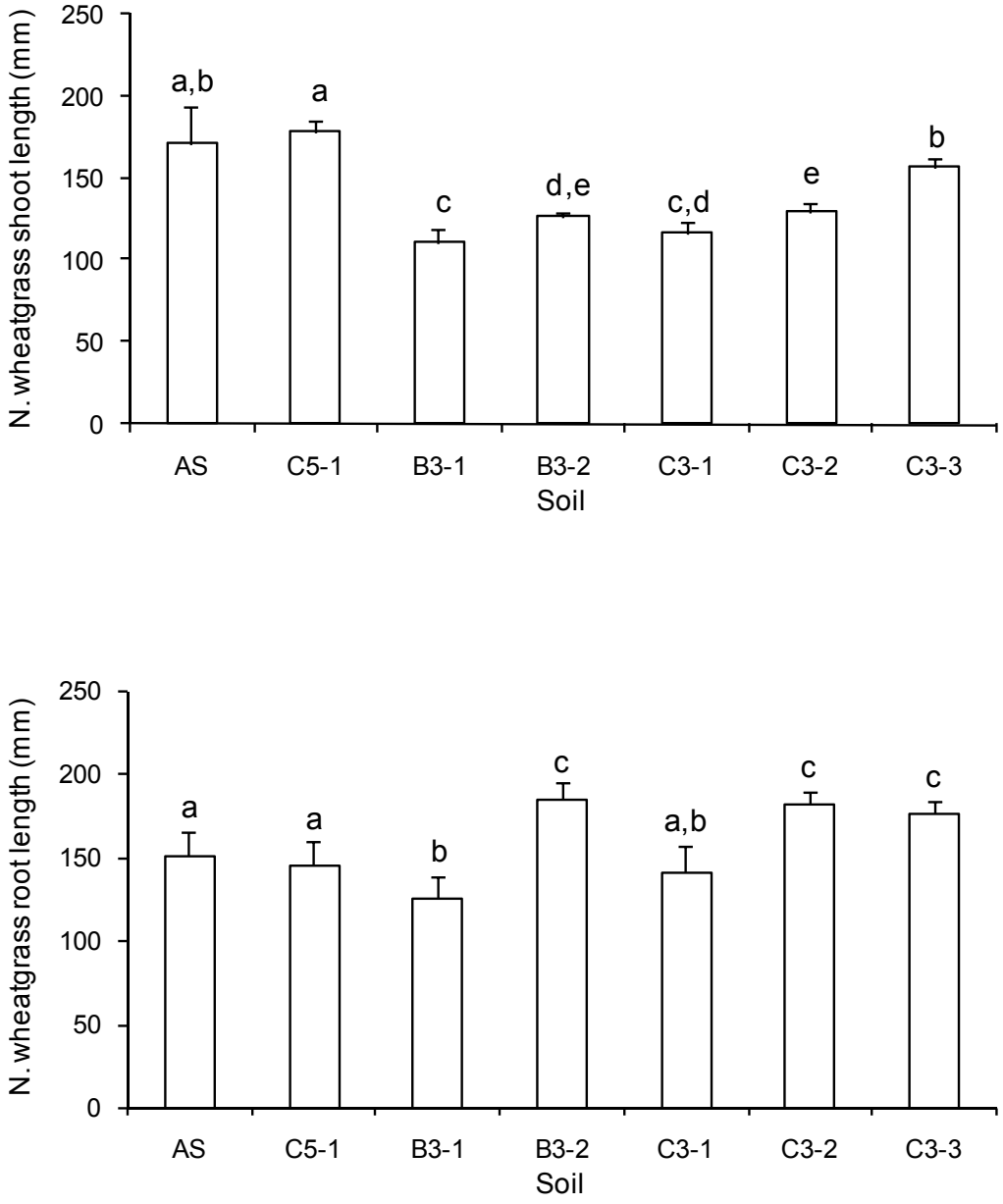

Fig. 3.2. Average northern wheatgrass shoot and root length following 21-days of exposure to site soils and AS (AS = artificial soil, C5-1 = reference site soil). Error bars indicate one standard deviation of the mean. Columns with the same letter indicate no significant differences at $p > 0.05$; $n = 5$.

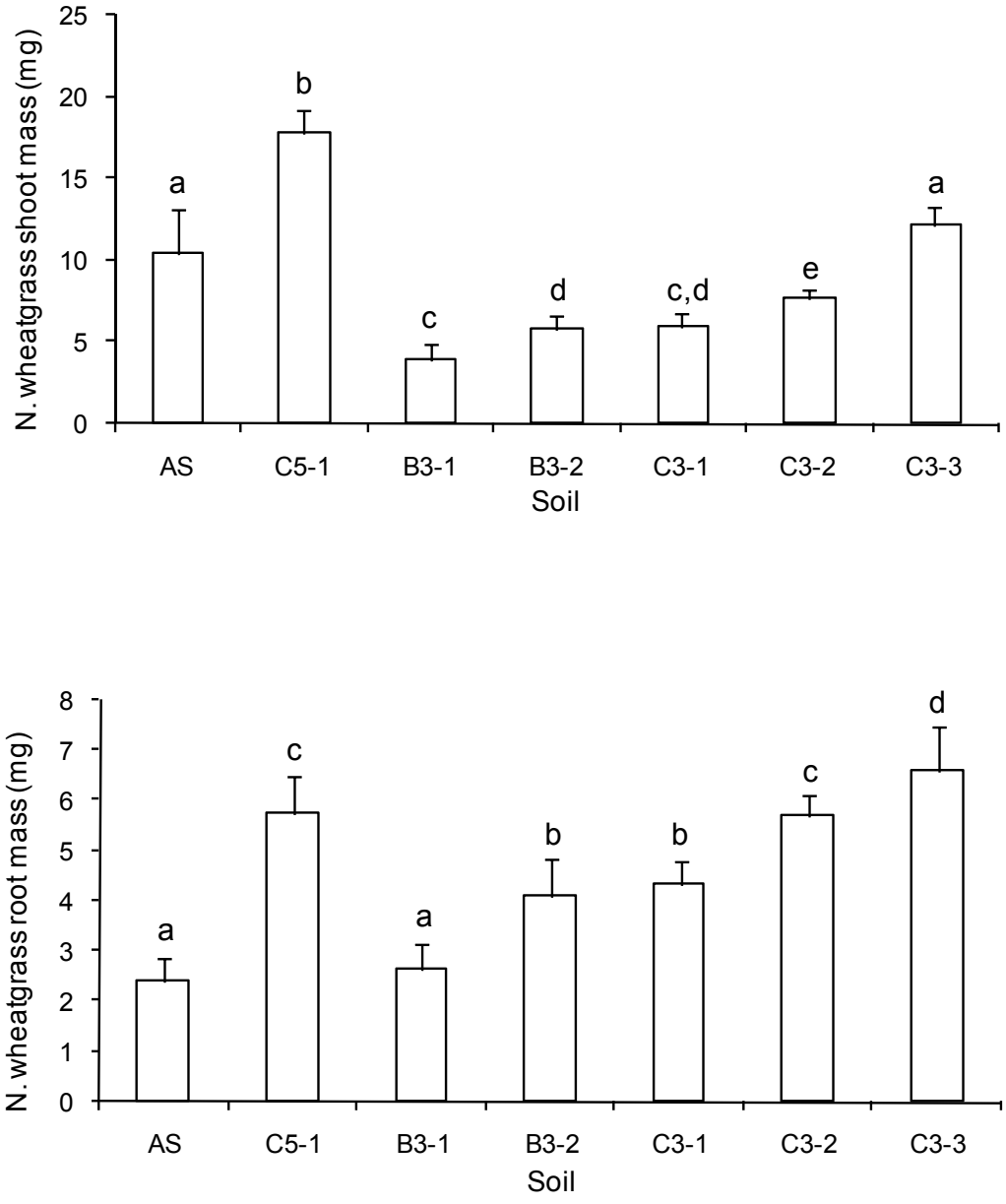

Fig. 3.3. Average northern wheatgrass shoot and root dry mass following 21-days of exposure to site soils and AS (AS = artificial soil, C5-1 = reference site soil). Error bars indicate one standard deviation of the mean. Columns with the same letter indicate no significant differences at $p > 0.05$; $n = 5$.

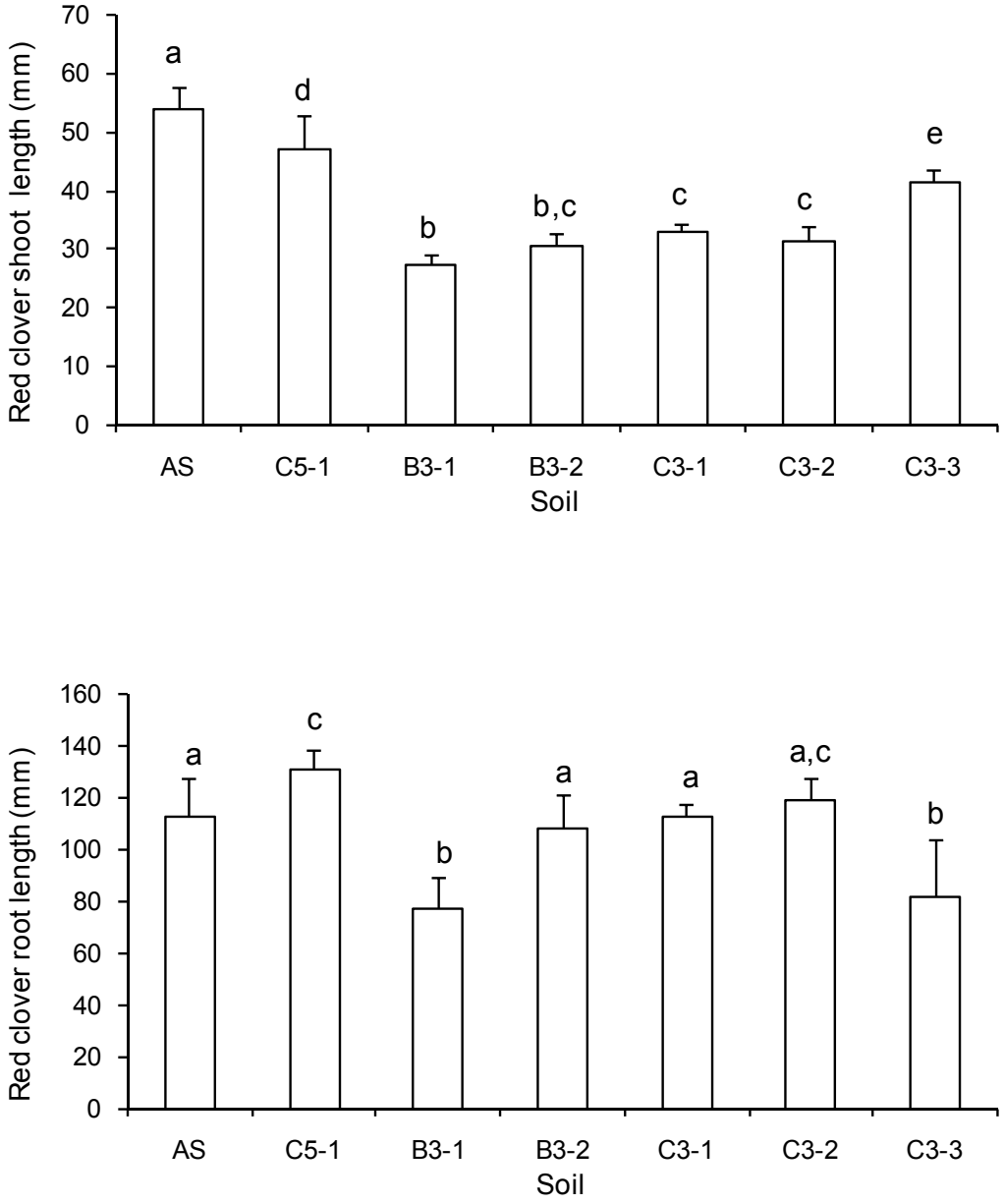

Fig. 3.4. Average red clover shoot and root length following 14-days of exposure to site soils and AS (AS = artificial soil, C5-1 = reference site soil). Error bars indicate one standard deviation of the mean. Columns with the same letter indicate no significant differences at $p > 0.05$; $n = 5$.

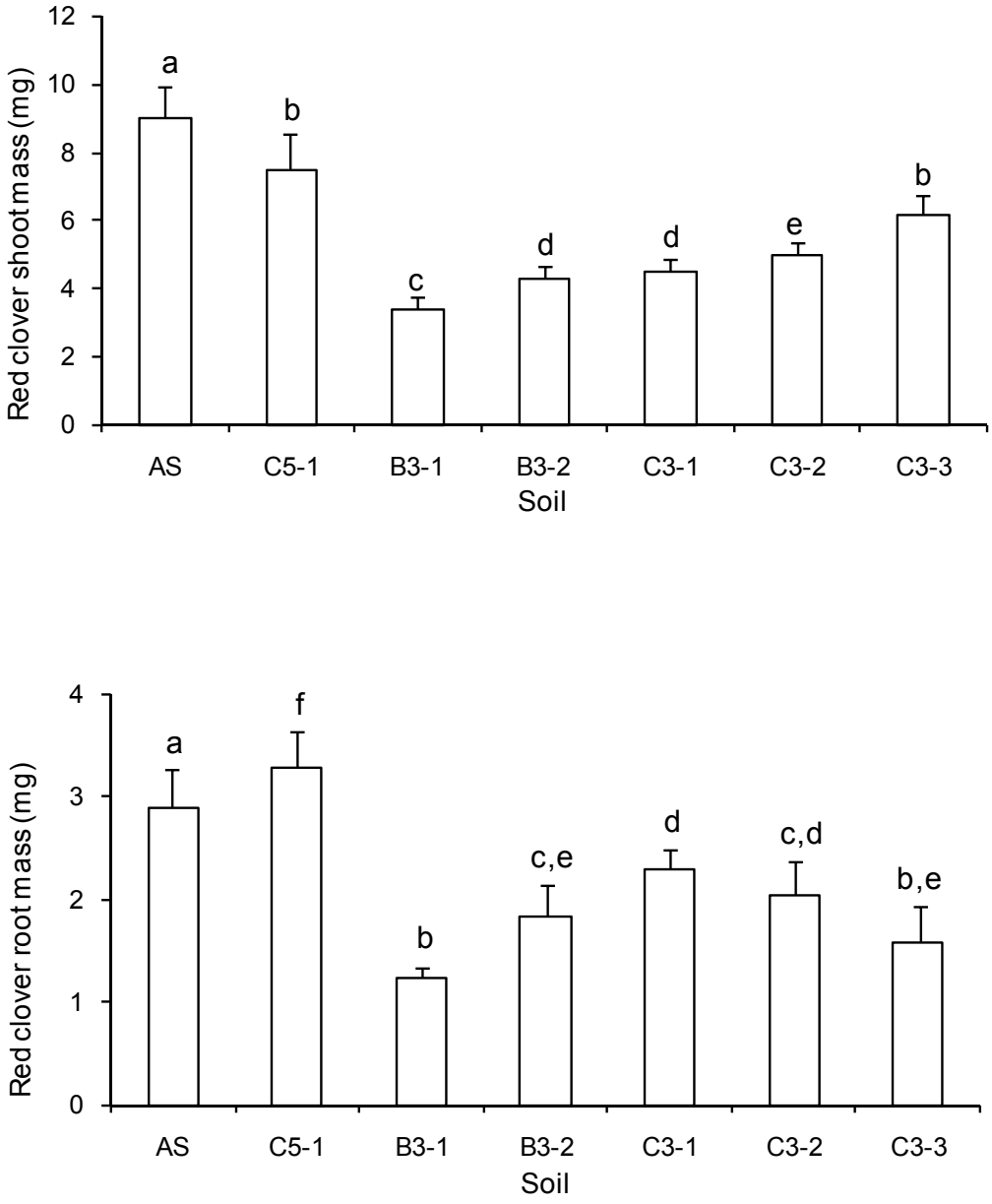

Fig. 3.5. Average red clover shoot and root dry mass following 14-days of exposure to site soils and AS (AS = artificial soil, C5-1 = reference site soil). Error bars indicate one standard deviation of the mean. Columns with the same letter indicate no significant differences at $p > 0.05$; $n = 5$.

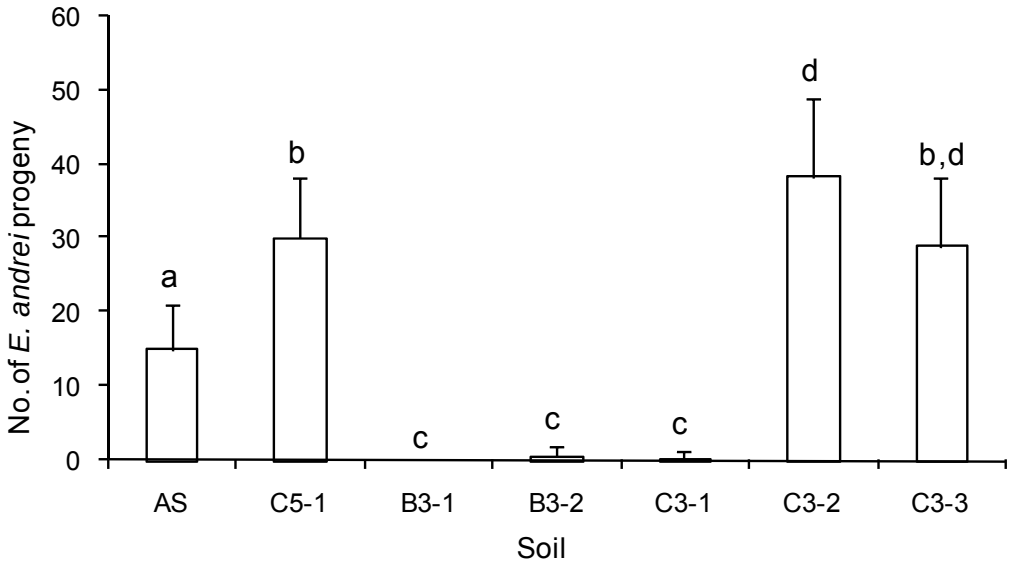

Fig. 3.6. Mean number of *Eisenia andrei* progeny produced following 63-days exposure to site soils and AS (AS = artificial soil, C5-1 = reference site soil). Error bars indicate one standard deviation of the mean. Columns with the same letter indicate no significant differences at $p > 0.05$; $n = 10$.

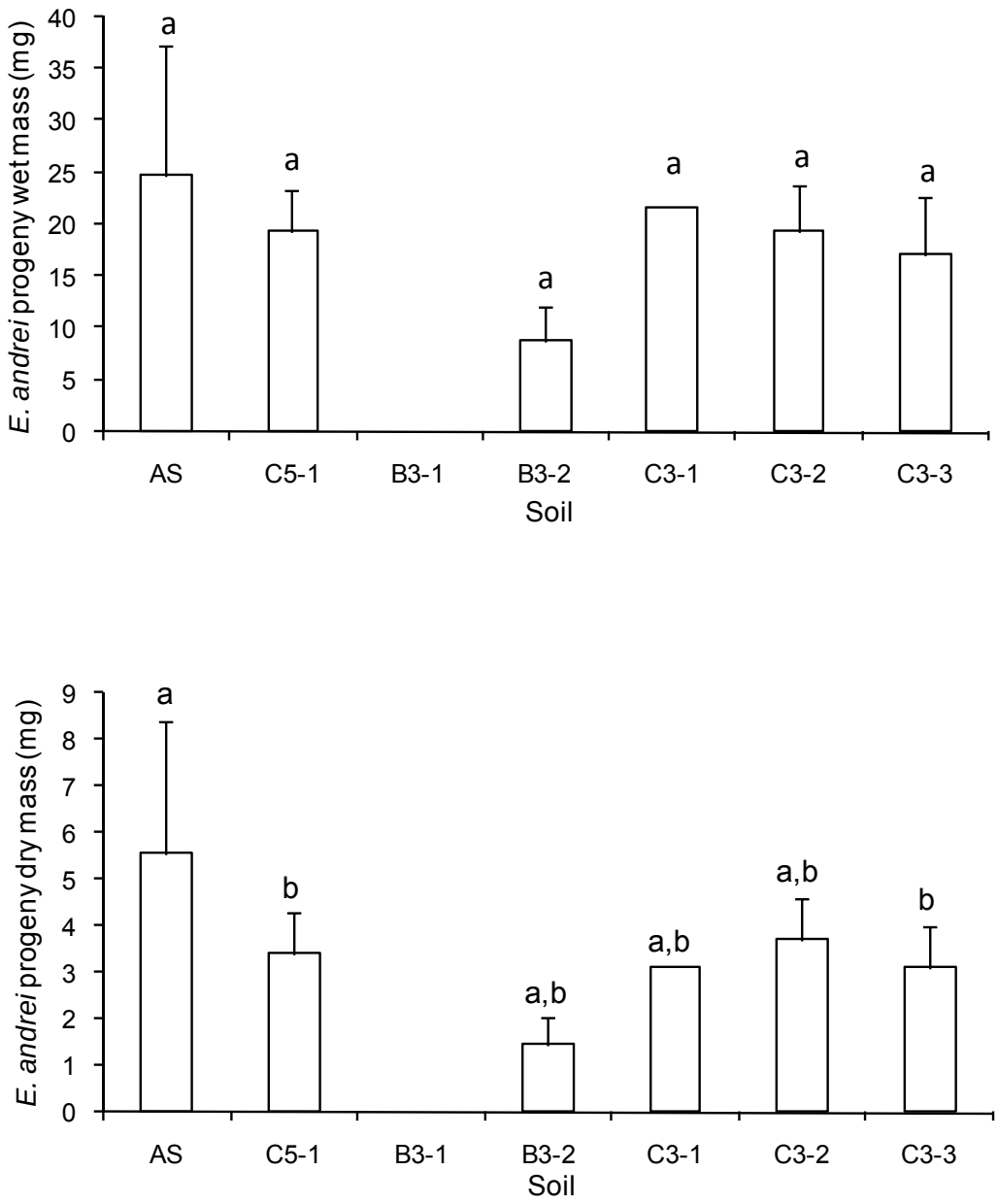

Fig. 3.7. Mean mass of *Eisenia andrei* progeny following 63-days exposure to site soils and AS (AS = artificial soil, C5-1 = reference site soil). Error bars indicate one standard deviation of the mean. Columns with the same letter indicate no significant differences at $p > 0.05$; n was as follows: AS, 10; C5-1, 9; B3-1, 0; B3-2, 3; C3-1, 1; C3-2, 10; and C3-3, 10.

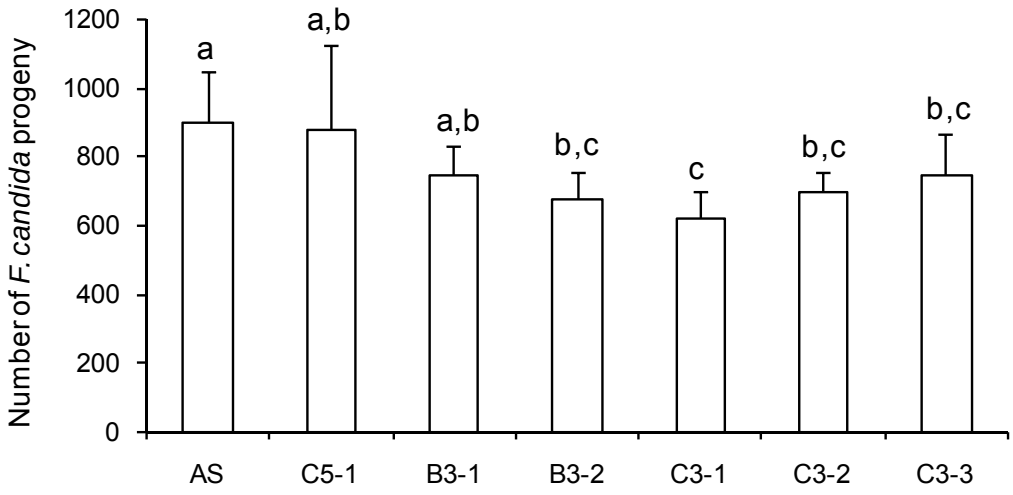

Fig. 3.8. Effects on survival and reproduction of *Folsomia candida* following 28-days exposure to site soils and AS (AS = artificial soil, C5-1 = reference site soil). Error bars indicate one standard deviation of the mean. Columns with the same letter indicate no significant differences at $p > 0.05$; $n = 5$.

3.3.6. Comparison of results

Table 3.5 shows the correlation between surrogate measures of bioavailability and biological responses. Regressions were only performed using effects data for which there were significant differences between at least two of the site soils. Multivariate regressions were used to assess the effectiveness of total, $CaCl_2$-extractable, and plant tissue concentrations for predicting adverse effects since concentrations of Cu and Zn did not autocorrelate to a high degree. Univariate regressions were used to assess the effectiveness of cyclodextrin and SEG-extractable, as well as, *E. andrei* tissue concentrations since concentrations of Cu and Zn autocorrelated (i.e., correlation coefficients were less than -0.8).

Table 3.5. Results of univariate and multivariate regressions for the site soils. Concentrations of metals in soils or biological tissues determined using each surrogate measure of bioavailability were compared with adverse effects data. Where concentrations of metals autocorrelate, the adjusted r^2 values of univariate regressions are shown; otherwise, multivariate adjusted r^2 and standard coefficient values are shown.

				Eisenia andrei effects				*Folsomia candida* effects	Northern wheatgrass effects				Red clover effects			
Measure	Regression	Value	Metal	No. of progeny	Progeny wet wt.	Progeny dry wt.	Day 14 weight change	No. of progeny	Shoot length	Root length	Shoot mass	Root mass	Shoot length	Root length	Shoot mass	Root mass
Total	M	Standard coefficient	Cu	-0.879^	-1.346^	-1.550^	-0.848^	-0.240	-0.893^	-0.471*	-0.917^	-1.005^	-0.885^	-0.628^	-1.006^	-0.779^
			Zn	0.263**	1.030*	1.244^	0.179	-0.359	0.112	0.622^	0.085	0.717^	0.286*	0.068	0.352^	0.142
		Adjusted r^2		0.617^	0.279^	0.366^	0.551^	0.216*	0.692^	0.274**	0.757^	0.812^	0.590^	0.310^	0.776^	0.483^
$CaCl_2$	M	Standard coefficient	Cu	-0.732^	-1.206^	-1.438^	-0.841^	-0.127	-0.666^	-1.038^	-0.556^	-0.897^	-0.535**	NA	-0.709^	-0.231
			Zn	-0.187*	0.605	0.831*	-0.088	-0.405	-0.166	0.852^	-0.295	0.298	-0.116	NA	-0.062	-0.278
		Adjusted r^2		0.721^	0.432^	0.512^	0.777^	0.185*	0.574^	0.730^	0.561^	0.544^	0.327^	0.103	0.526^	0.148*
Cyclodextrin	U	Adjusted r^2	Cu	0.532^	0.344^	0.350^	0.417^	0.000	0.486^	0.065	0.556^	0.666^	0.508^	0.383^	0.682^	0.557^
			Zn	0.560^	0.374^	0.391^	0.430^	0.000	0.466^	0.059	0.538^	0.666^	0.489*	0.361^	0.664^	0.527^
SEG	U	Adjusted r^2	Cu	0.602^	0.337^	0.332^	0.486^	0.168	0.632^	0.226**	0.632^	0.760^	0.562^	0.252^	0.760^	0.384^
			Zn	0.723^	0.363^	0.373^	0.764^	0.096	0.640^	0.207**	0.614^	0.583^	0.442^	0.174*	0.649^	0.232^
Eisenia andrei tissue concentration	U	Adjusted r^2	Cu	0.012	0.117*	0.082	0.000	0.002	ND	ND	ND	ND	ND	ND	ND	ND
			Zn	0.000	0.000	0.000	0.000	0.000	ND	ND	ND	ND	ND	ND	ND	ND
Red clover shoot tissue concentration	U	Standard coefficient	Cu	ND	ND	ND	ND	ND	-0.480^	-0.589^	-0.497^	-0.866^	-0.504^	-0.603^	-0.678^	-0.619^
			Zn	ND	ND	ND	ND	ND	-0.307	0.720^	-0.401**	0.257*	-0.243	-0.194	-0.158	-0.31*
		Adjusted r^2		ND	ND	ND	ND	ND	0.370^	0.579^	0.493^	0.658^	0.343^	0.433^	0.516^	0.566^
Red clover root tissue concentration	U	Standard coefficient	Cu	ND	ND	ND	ND	ND	0.152	-0.867^	0.227	-0.509**	0.235	-0.024	-0.017	0.228
			Zn	ND	ND	ND	ND	ND	-0.716^	-0.543^	-0.725^	-0.882^	-0.581^	-0.578**	-0.801^	-0.554^
		Adjusted r^2		ND	ND	ND	ND	ND	0.636^	0.464^	0.752^	0.480^	0.518^	0.268**	0.598^	0.469^

*: $p < 0.05$

**: $p < 0.01$

^: $p < 0.005$

M: Multivariate

U: Univariate

NA: Not applicable because multivariate regression was not significant ($p > 0.05$)

ND: Not determined

3.3.6.1 Comparison of measures of bioavailability with effects to invertebrates

In general, the correlations of all surrogate measures of bioavailability with *E. andrei* effects data were fairly comparable, with the exception of *E. andrei* tissue residues. All extraction tests significantly ($p < 0.005$) correlated with adverse effects to *E. andrei*. The SEG test (Cu and Zn adjusted r^2 values of 0.602 and 0.723 respectively) and $CaCl_2$ extraction (multivariate adjusted $r^2 = 0.721$) were the best methods for predicting the number of progeny produced. No measures were adequately predictive of progeny wet mass, and the $CaCl_2$ extraction was a fair predictor of progeny dry mass (adjusted $r^2 = 0.512$). Day 14 weight change was best correlated with the SEG test (Cu and Zn adjusted r^2 values of 0.486 and 0.764 respectively) and $CaCl_2$ extraction (multivariate adjusted $r^2 = 0.777$). The cyclodextrin and SEG extractions indicated that both Cu and Zn were positively correlated with adverse effects, whereas the total and $CaCl_2$-extractable concentrations indicated that only Cu was positively correlated with adverse effects. Since cyclodextrin and SEG-extractable concentrations or Cu and Zn autocorrelated to high degree, it is not surprising that both metals were predictive of effects for both tests. Based on the data from the $CaCl_2$ extraction, Cu could be the primary metal causing toxicity to earthworms.

No surrogate measure of bioavailability was predictive of effects observed for *F. candida*. Although $p < 0.05$ for some measures, adjusted r^2 values were < 0.5.

3.3.6.2 Comparison of measures of bioavailability with effects to northern wheatgrass

All measures of bioavailability significantly predicted growth effects on northern wheatgrass shoots ($p < 0.005$). However, the relative contribution of Cu and Zn concentrations varied greatly among measures. For example, analyses of $CaCl_2$-extractable metal concentrations indicated that Cu was the sole metal negatively correlated with northern

wheatgrass root length; however, analyses of red clover root tissues indicated that concentrations of Zn, not Cu, were negatively correlated with northern wheatgrass root length.

Total, red clover root tissue, and SEG-extractable concentrations correlated best with northern wheatgrass shoot length and mass. However, these measures were not necessarily predictive of effects on root length and mass. The best predictor of northern wheatgrass root length and root mass was $CaCl_2$-extractable metals and total metals, respectively. It is difficult to discern the best predictor of all effects on northern wheatgrass based on these correlations; however, it is evident that none of the surrogate measures of bioavailability had greater predictive power than total metal concentrations for all effects. As well, the metal primarily indicative of adverse effects was not consistent among the measures of bioavailability tested; neither within a single measure of bioavailability (e.g., red clover root tissue concentrations) nor across endpoints.

3.3.6.3 Comparison of measures of bioavailability with effects to red clover

All measures of bioavailability significantly predicted red clover shoot effects ($p < 0.005$). Total, SEG-extractable, red clover root tissue, and cyclodextrin-extractable concentrations were negatively correlated with red clover shoot length, in decreasing order of model fit, respectively. All measures were good predictors of shoot mass, and shoot mass correlated best with total metal concentrations. No measures adequately predicted red clover root length (adjusted $r^2 < 0.5$) although correlation was significant for all measures ($p < 0.05$) except the $CaCl_2$ extraction. All measures significantly correlated with red clover root mass, but only red clover shoot concentrations and cyclodextrin-extractable metals correlated well (adjusted $r^2 > 0.5$). Ultimately, no measure of bioavailability correlated better with adverse effects to red clover than total metal concentrations in soil. Total metal concentrations indicated that toxicity might be attributed to Cu in the soils ($p < 0.005$ for all biological endpoints).

3.3.7. Summary of results

Extractable concentrations of Cu and Zn were highly autocorrelated for cyclodextrin and SEG tests. Accumulation of Cu in red clover and earthworm tissue was soil-dependent. Uptake and elimination of Zn may have been regulated by earthworms. In general, adverse effects to invertebrates and plants were greatest in soils B3-1, B3-2, and C3-1. For comparisons of the results of the measures of bioavailability with the results of the toxicity tests, the $CaCl_2$ extraction and the SEG test were correlated with effects to *E. andrei*, and no measure was a good predictor of effects to *F. candida.* Adverse effects to plants were generally best correlated with total metal concentrations in the soils.

3.4. Discussion

LC50s reported for *E. fetida* were 836 mg/kg and >1078 mg/kg following acute exposure to AS spiked with Cu and Zn, respectively [5]. The maximum exposure concentrations in the soils in this Chapter were two times higher than these EC50s, yet no adult earthworm mortality was recorded for *E. andrei* following 35-days of exposure to the soils, suggesting that site-specific EC50s were several times higher than those obtained using freshly spiked soil media. These results correspond with those reported by Spurgeon and Hopkin [5] and illustrate the potential problems encountered when using total metal concentrations that are unrealistically readily available (i.e., freshly spiked with soluble metal salts) in soil to set Tier 1 ecological benchmarks. Using freshly spiked media and soluble metal salts to derive benchmarks does not allow physical process such as ageing, weathering, sequestration, adsorption, and degradation to occur and may not reflect site conditions. Generally, sequestered and recalcitrant residuals are not readily bioavailable to ecological receptors. Metals will rarely, if ever, be 100% bioavailable in site soils [33] yet ecological benchmarks are often derived using toxicity tests where a major fraction of the contaminant is bioavailable.

Concentrations of Cu and Zn mobilised by the chemical extractions applied to soils described herein were present in extracts well above analytical detection limits. Concentrations of Cu extracted using the 0.5 M $CaCl_2$ solution were greater than those extracted using cyclodextrin solution and the SEG test. This is contrary to results obtained in Chapter 2, where concentrations of Cu in $CaCl_2$ extracts were below detection limits and below those in cyclodextrin and SEG solutions. $CaCl_2$ solution is a "soft" extraction method considered to represent the labile fraction of metals in soil (i.e., it provides an indication of the amount of metal that can be desorbed, or is water-soluble and exchangeable) [23, 39]. Complexing agents, such as cyclodextrin and the SEG formulation, are expected to measure the fraction of metals bound to soil OM and, for the latter, possibly mobilised in an earthworm's GI tract with the aid of digestive enzymes and microbial processes. Since more Cu was solubilised by $CaCl_2$ solution than the other extraction methods we used, metals in these soils were relatively water-soluble.

Uptake and elimination of Zn is controlled by earthworms [39]. *E. andrei* tissue concentrations of Zn throughout exposure to these soils were between approximately 100 mg/kg to 150 mg/kg dry worm tissue regardless of exposure concentration. This is similar to previous investigations where internal *Eisenia* spp. concentrations of Zn ranged from 100 mg/kg to 200 mg/kg [83], 79 mg/kg to 151 mg/kg [18], approximately 100 mg/kg [17], and 75 mg/kg to 120 mg/kg [96] across a range of soil concentrations. However, Hobbelen *et al*. [39] observed concentrations of Zn as high as 1871 mg/kg in *L. rubellus* and *Apporectodea caliginosa* earthworms, and no regulation of Zn was observed in *L. terrestris* [92]. Therefore, bioaccumulation of Zn varies among species. It is important to note that although Zn does not bioaccumulate to any appreciable degree in *E. andrei*, elevated soil concentrations are still toxic [83] and body concentrations are inadequate predictors of toxicity.

Internal Cu concentrations in earthworms from this study were similar to levels previously reported in *Eisenia* spp. (e.g., [18]) and were different among individuals exposed to

different soils. All adult *E. andrei* survived exposure to amended soils used for the toxicity test, but some adult mortality was observed during the bioaccumulation test with unamended soils. Tissue concentrations of Cu between 50 to 60 mg/kg are indicative of earthworm mortality [14]. *E. andrei* tissue concentrations exceeded 50 mg/kg in only one soil, B3-2. However, no mortality was observed in worms exposed to the amended or unamended B3-2 soil, suggesting that the critical body residues observed by Ma *et al*. [14] for *L. rubellus* and *A. caliginosa* are not applicable to *E. andrei*.

The differences in adult mortality observed between the earthworm reproduction toxicity test and the bioaccumulation test suggest that the lower pH of the unamended soils may have influenced earthworm survival. *E. andrei* are adversely affected by acidic soils [41, 74] and Cu is more bioavailable in soils with low pH (i.e., 4.3 versus 6.0) [14]. Adjusting soil pH has a significant effect on bioavailability and toxicity of metals [40, 73]. In this case, it is unclear whether the adverse effects observed in unamended soils are attributable to low pH directly or indirectly (i.e., through the increase of soluble metals in soils). The effect of adjusting the soil pH (by amendment with $CaCO_3$) on adverse effects to earthworms and plants was not quantified in this study. However, since the primary objective was to relate surrogate measures of bioavailability to standard toxicity tests, and pH adjustment was necessary to carry out the ecotoxicity tests so that pH itself was not causing toxicity to test organisms, the amendment of soils for earthworm and plant toxicity testing was justifiable. Alternatively, test species tolerant of low soil pH could be used in future tests.

The effects of nutrient availability (e.g., phosphorus and nitrogen) and soil physico-chemical characteristics are not accounted for by surrogate measures of bioavailability. These factors could have a profound impact on the fitness of test organisms. However, it is assumed that the biological responses observed in this study are attributed primarily to exposure to bioavailable metals.

Earthworm tissue concentrations were not predictive of the effects of test soils to invertebrates, but chemical extractions were predictive of some earthworm biological responses. Using earthworm tissue concentrations to predict toxic effects of metals is problematic in ecological risk assessment [79, 82, 83]. Essential metals including Cu and Zn are regulated with the aid of metallothioneins in earthworms [17]. Internal body concentrations of metals may not correlate with fractions extracted by solutions such as $CaCl_2$ [23] since regulation is not accounted for by chemical measures of bioavailability. In some species, external concentrations may be better predictors of toxicity [50], such as those which were determined using chemical measures of bioavailability. The $CaCl_2$-extraction and SEG test were best correlated with adverse effects to earthworms. While these two extractions were expected to release different fractions of metals from soil, perhaps the relative contribution of metals, in the soluble pool and mobilised in the GI tract, to toxicity is similar. Otherwise, the enzymatic activity of the SEG solution may not sufficiently release bound metals from these highly organic soils, and instead the formulation acts as a simple ionic solution, releasing easily labile metals similar to the $CaCl_2$ solution. However, the fact that both SEG-extractable Cu and Zn positively correlated with effects to earthworms while only the $CaCl_2$-extractable Cu correlated with *E. andrei* effects, as well as, that extract concentrations of Cu and Zn differed between the two tests, suggests that the two methods might extract different fractions of metals. The cyclodextrin extraction was the least predictive chemical measure of bioavailability to earthworms, as has been noted for other complexing agents (see [23]).

F. candida was the only species exposed to unamended soils. No surrogate measure of bioavailability predicted adverse effects to this collembola species. Except for soil C3-1, no soils had a significant effect on *F. candida* biological responses. *F. candida* is resilient to high concentrations of Cu and Zn in these soils, even when soil conditions are acidic.

Correlations between plant tissue resides and biological endpoints are possible but internal levels of Cu and Zn might be regulated, making correlation challenging [51]. Although red clover tissue concentrations were predictive of effects to the plant species, adverse effects to plants were generally best described by total concentrations in soil. This contradicts findings of other researchers [7, 51, 73] who recommend weak chemical extractions or porewater concentrations to measure metal bioavailability to plants. However, those researchers based their recommendation on tests using soils with a wide range of physico-chemical characteristics, many of which were not highly organic soils. For the soils used in the Chapter, both the cyclodextrin and SEG extractions correlated better with plant effects than the $CaCl_2$ extractions, and were, in some cases, better correlated than biological measures of bioavailability. It is possible that a greater proportion of metal exposure to plants was to Cu weakly-bound to organic material, and this Cu was not released by extraction with $CaCl_2$.

Several researchers have recognised that a method to estimate bioavailability using a chemical extraction would be an invaluable screening tool at contaminated sites (e.g., [33, 47, 59]). In Chapter 2, surrogate biological and chemical measures of bioavailability were investigated. The SEG test was the best predictor of metal bioavailability to ecological receptors, using soils with relatively low OM content and contaminated with Cu, Pb and Zn. Generally, adverse effects were best correlated with SEG-extractable Cu in those soils. In this Chapter, the performance of the same measures of bioavailability and metal concentrations in plant tissue using a set of soils with different physico-chemical characteristics was investigated; however, the same conclusion cannot be drawn. Although the SEG test, as well as other extraction techniques and plant tissue concentrations, correlated well with many toxicological endpoints, the correlations between total metal concentrations and plant biological endpoints were often better. The SEG test and $CaCl_2$ extractions were, however, the best predictors of adverse effects to earthworms in this Chapter.

3.5. Conclusion

It is difficult to interpret toxicological parameters derived from field studies due to co-contamination and variations in soil properties. However, results obtained from tests with field-collected site soils are more applicable to a contaminated site as they are more realistic than tests with chemically spiked artificial or uncontaminated field soils [42].

Evidence of porewater uptake is circumstantial for metals [51]. The relative toxicity and bioavailability of Cu and Zn in highly organic mining soils is receptor-dependent. Chemical measures of bioavailability are better predictors of adverse effects to invertebrates than contaminant residues in *E. andrei* tissue, and red clover tissue concentrations and chemical extractions are both reliable indicators of plant effects and metal bioavailability. No single chemical extraction technique can be recommended as a surrogate measure of bioavailability to all ecological receptors, but $CaCl_2$ and SEG extractable metals may be the best indicators of Cu and Zn mixture toxicity to *E. andrei* in highly organic soils. Generally, total metal concentrations are the best predictors of adverse effects to plants in these soils. Based on these results and those in Chapter 2, the SEG test may be a good indication of metal toxicity at contaminated sites with varying soil physico-chemical characteristics.

3.6. Summary

The soil contact exposure pathway can be the main driver of ecological risk assessments. There is currently no standard method to measure bioavailability of metals in soil to ecological receptors, yet the influence of metal bioavailability on toxicity has been known for decades and is a major issue in ecological risk assessment. Bioavailability can be drastically altered by varying soil characteristics at different sites, yet Tier 1 ecological benchmarks are often derived on a total concentration basis. The SEG test has been shown to correlate with adverse effects to ecological receptors in soils composed primarily of fill material. A $CaCl_2$

extraction, cyclodextrin extraction, SEG test, earthworm kinetic bioaccumulation test, and metal residues in plant tissues were compared to a battery of invertebrate and toxicity tests using mining soils with high organic matter content co-contaminated with Cu and Zn. *E. andrei* tissue concentrations of Cu and Zn were not predictive of adverse effects to invertebrates. All chemical measures of bioavailability correlated with several biological responses; however, $CaCl_2$-extractable Cu and SEG-extractable Cu and Zn best predicted adverse effects to *E. andrei*. Generally, total Cu concentrations in soil best predicted plant growth. Overall, a chemical measure was the best predictor of effects to each organism, although the exact measure was dependent on organism and endpoint. Chemical extraction techniques provide relatively quick, inexpensive indicators of essential metal bioavailability compared to biological measures. The pool of metals extracted by the SEG formulation is expected to represent that which is mobilised in the GI tract of *E. andrei*; however, this theory needs further investigation.

CHAPTER 4

CONCLUSIONS

The primary conclusion drawn from Chapter 2 was that the SEG test showed the most promise for predicting bioavailability of Cu, Pb, and Zn mixtures to terrestrial invertebrate and plant species when compared to earthworm bioaccumulation tests, $CaCl_2$ extractions, cyclodextrin extractions, and total metal content in soil. Generally, adverse effects were correlated with the bioavailable fraction of Cu. However, this was the first time that this test had ever been employed to assess the bioavailability of mixtures of contaminants in soil to ecological receptors and further evaluations were needed to assess its suitability for soils with different physico-chemical characteristics.

In Chapter 3, the same measures of bioavailability were applied to highly organic soils contaminated with Cu and Zn and, in addition, tissue metal residues in red clover were measured. Results indicated that for highly organic soils no single measure of bioavailability was consistently a better predictor of adverse effects to plants. The SEG test and $CaCl_2$ extraction were (equally) the most predictive of effects to earthworms.

To determine whether any measures of bioavailability were predictive of adverse effects in both sets of soils combined, data from Chapter 2 and Chapter 3 were pooled to conduct statistical analyses to compare measures of bioavailability (i.e., total Cu and Zn concentrations, $CaCl_2$-extractable Cu and Zn, cyclodextrin-extractable Cu and Zn, SEG-extractable Cu and Zn, and *E. andrei* tissue concentrations of Cu and Zn) with the common biological responses (i.e., *E. andrei*, *F. candida*, and northern wheatgrass toxicity data). Results of these regressions are provided in Table 4.1.

Table 4.1. Results of multivariate regressions for all site soils from Chapter 2 and Chapter 3. Concentrations of metals in soils or biological tissues determined using each surrogate measure of bioavailability were compared with adverse effects data.

				Eisenia andrei effects			*Folsomia candida* effects	Northern wheatgrass effects			
Measure	Regression	Value	Metal	No. of progeny	Progeny wet wt.	Progeny dry wt.	No. of progeny	Shoot length	Root length	Shoot mass	Root mass
Total	M	Standard coefficient	Cu	-0.650^	NA	NA	-0.364*	-0.584^	-0.049	-0.703^	-0.448*
			Zn	0.475^	NA	NA	-0.322*	0.166	0.661^	0.254	0.850^
		Adjusted r^2		0.175^	0.038	0.042	0.389^	0.195^	0.367^	0.268^	0.337^
$CaCl_2$	M	Standard coefficient	Cu	-0.812^	NA	NA	0.082	NA	-0.957^	NA	-0.684^
			Zn	0.497^	NA	NA	-0.630^	NA	1.400^	NA	1.176^
		Adjusted r^2		0.271^	0.000	0.000	0.293^	0.000	0.682^	0.000	0.527^
Cyclodextrin	M	Standard coefficient	Cu	-2.692^	-0.859**	0.050	1.113*	-1.145*	-2.361^	-2.159^	-3.212^
			Zn	2.389^	.869**	-0.715*	-1.604*	0.736	2.723^	1.699^	3.295^
		Adjusted r^2		0.429^	0.090^	0.618*	0.316^	0.182^	0.464^	0.393^	0.582^
SEG	M	Standard coefficient	Cu	-0.672^	-0.296*	NA	-0.132	-0.411^	-0.466^	-0.564^	-0.677^
			Zn	-0.061	0.071	NA	-0.449^	-0.276*	0.493^	-0.230	0.383^
		Adjusted r^2		0.480^	0.065*	0.049	0.240^	0.317^	0.225^	0.463^	0.353^
Eisenia andrei tissue concentration	M	Standard coefficient	Cu	-0.369*	NA	NA	-0.183	ND	ND	ND	ND
			Zn	0.201	NA	NA	-0.324	ND	ND	ND	ND
		Adjusted r^2		0.046*	0.000	0.027	0.200^	ND	ND	ND	ND

*: $p < 0.05$

**: $p < 0.01$

^: $p < 0.005$

M: Multivariate

NA: Not applicable because multivariate regression was not significant ($p > 0.05$)

ND: Not determined

None of the Cu and Zn concentrations autocorrelated, so univariate regressions were not needed. For the combined data no measures of bioavailability were good predictors of invertebrate effects (i.e., adjusted $r^2 < 0.5$). The SEG test was the best predictor of *E. andrei* progeny production ($p < 0.005$, adjusted $r^2 = 0.480$), followed by the cyclodextrin extraction. However, the cyclodextrin extract concentrations were highly variable for the tests described in Chapter 2 and confidence in the regressions is low. Total concentrations were the best indicators of adverse effects to *F. candida*, but the correlation was not ideal (i.e., adjusted $r^2 = 0.389$). Northern wheatgrass root endpoints were best correlated with $CaCl_2$-extractable metals, with bioavailable Cu positively correlated with adverse effects. However, $CaCl_2$-extractable metals were not indicative of effects on shoots. SEG-extractable metals correlated best with northern wheatgrass shoot endpoints, but their predictive power was low (i.e., adjusted $r^2 < 0.5$).

The surrogate measures applied in this thesis were suitable for assessing the bioavailability of Cu and Zn, and sometimes Pb in field soils. However, the fit of each measure to ecotoxicity data was organism and response dependent and no measure could adequately predict effects in all instances. The SEG test was consistently the best predictor of *E. andrei* progeny production, and toxicity might be attributed to the bioavailable Cu fraction in all soils. The $CaCl_2$ extraction, which is commonly used to assess metal bioavailability to plants, was not the best predictor of plant effects, particularly for soils used in Chapter 2. Cu was present in several $CaCl_2$ extracts at concentrations below the analytical method detection limit, which likely affected the predictive power of this extraction test.

The bioavailable metal pool differed between the two sets of soils used in each experiment. Physico-chemical characteristics of the soils themselves, as well as, the contamination type and source are likely the primary factors governing the bioavailability of metals. Soils used in Chapter 2 consisted mainly of fill material with pH greater than neutral.

Soils used for testing in Chapter 3 were highly organic, acidic forest soils from a former Cu and Zn mining site. Therefore, it is not surprising that the relative strength of each test for predicting bioavailability of metal mixtures differed when applied at the two different sites.

The different results and conclusions drawn in Chapter 2 and Chapter 3 and even from the combined data illustrate the difficulty surrounding the estimation of metal bioavailability at different sites. For this reason a suite of tools to estimate bioavailability would be very useful to apply at sites with different contamination issues and soil types. The surrogate measures of bioavailability used in this thesis could be applied individually at a contaminated site; it is important to understand the consequences of using just one measure to predict bioavailability, and the research presented throughout the second and third chapters emphasises that the laboratory test used to estimate bioavailability should be carefully selected with the understanding that the tool itself could significantly impact the conclusions reached.

In order for a surrogate measure of bioavailability to be used as a replacement of ecotoxicity tests, or as a screening tool at contaminated sites, extensive validation with biological responses measured in toxicity tests is required. Total metal concentrations may be the best indicators of bioavailability to plants at forested sites with high soil OM content, and the SEG test may be the best predictor of bioavailability to plants at sites consisting primarily of fill material. The SEG test is the best predictor of adverse effects to earthworms in both sets of soils. The dataset generated in Chapter 2 and Chapter 3 is simply too small to conclusively state that the SEG test is applicable to all soils, or even a set of soils with similar characteristics. In total, eleven site soils were used in this preliminary validation. The SEG should be validated with several times this number of soils; however, this was logistically impractical. However, the further validation of this method is an exciting research project that could have major implications on ecological risk assessment.

This thesis contributes to previous validation studies using biological and chemical measures of bioavailability, and could act as a starting point for further validation of the novel SEG test for assessing contaminant bioavailability to both invertebrates and plants. This research also indicates that chemical measures can be used to predict bioavailability of metal mixtures in soil. Typically, chemical measures are faster and cheaper methods than biological measures and do not require as many resources, including the number of technicians and quantity of contaminated soil needed. Further studies are needed to validate the SEG test and to determine the pool(s) of metals solubilised by the SEG formulation.

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APPENDIX A
PRELIMINARY CHEMICAL EXTRACTION TESTING

A.1. Development of the calcium chloride extraction

A range of $CaCl_2$ concentrations was used to extract metals from contaminated field soils. Preliminary testing was carried out using some of the soils described in Chapter 2 to determine a concentration suitable for the definitive tests (i.e., so that metals were detected above detection limits in extracts). Preliminary $CaCl_2$ extractions were performed using soils B, C, and E following the general method described in Chapter 2. However, a range of literature reported $CaCl_2$ concentrations (i.e., 0.01 M, 0.1 M, and 1 M) were used.

Field soils were sieved to ≤2 mm and air-dried for >72 hours. 10 ± 0.05 g of each soil were shaken at 120 rpm with 100 mL of each $CaCl_2$ solution for 2 hours in glass centrifuge tubes on a rotary platform shaker. Tubes were removed and centrifuged at 1,800 g for 10 minutes. Supernatant of each tube was extracted using 0.45 µm cellulose acetate filters attached to disposable 20-cc syringes and filtered into clean 15-mL centrifuge tubes. Each 10-mL extraction fluid sample was acidified with 0.1 mL of 1 M HCl and stored at 4ºC. Extracts were submitted to ALS Laboratory Group in Waterloo, Ontario, Canada for metal analyses within one week of extraction.

The results of the preliminary extractions are provided in Fig. A.1, Fig. A.2, and Fig. A.3. Solutions of 0.01 M and 0.1 M $CaCl_2$ were not more efficient at extracting metals than DI water. The solution of 1 M $CaCl_2$ extracted more metals than the other $CaCl_2$ concentrations and DI

water. A 1 M $CaCl_2$ solution is rarely used in the literature and may have too high of an ionic strength to represent the easily labile fraction of metals in soil. Based on recommendations of other researchers, as well as, the large amount of $CaCl_2$ reagents that would be required to perform definitive extractions in Chapter 2 and Chapter 3, a $CaCl_2$ concentration between 0.1 M and 1 M was desired. A concentration of 0.5 M $CaCl_2$ has been used in other investigations, and was selected as a suitable concentration for the definitive extractions.

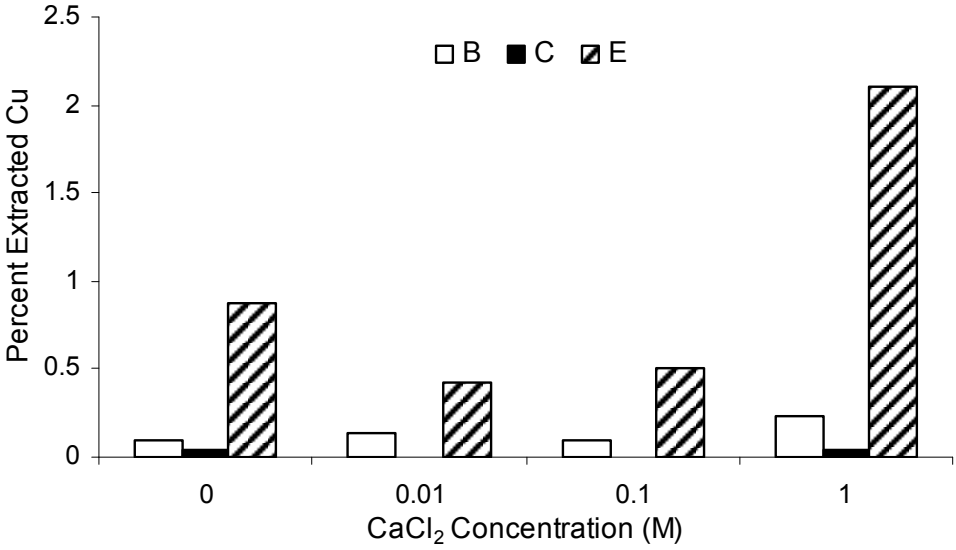


Fig. A.1. Percent of total Cu that was extracted from soils B, C, and E using different concentrations of $CaCl_2$. Where no column is visible, the element was present below detection limits in extracts; *n* =1.

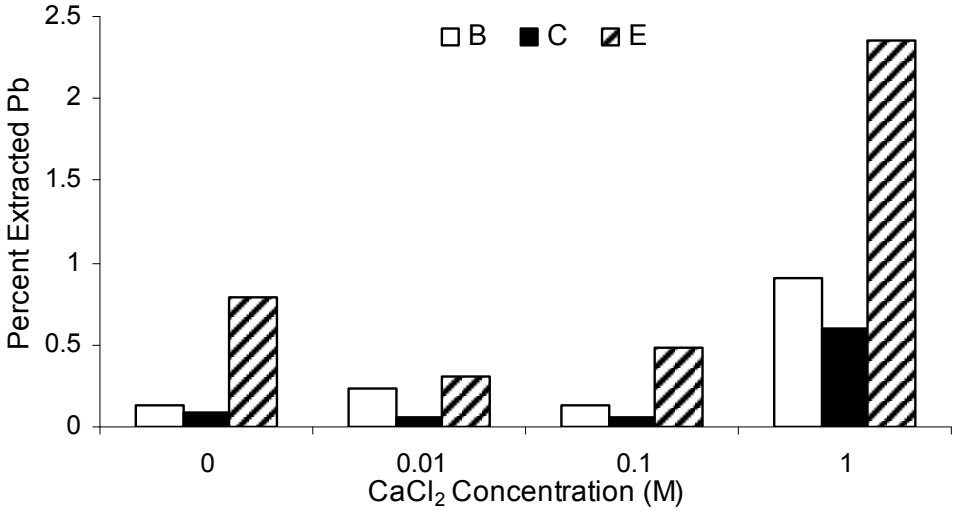


Fig. A.2. Percent of total Pb that was extracted from soils B, C, and E using different concentrations of $CaCl_2$. Where no column is visible, the element was present below detection limits in extracts; *n* =1.

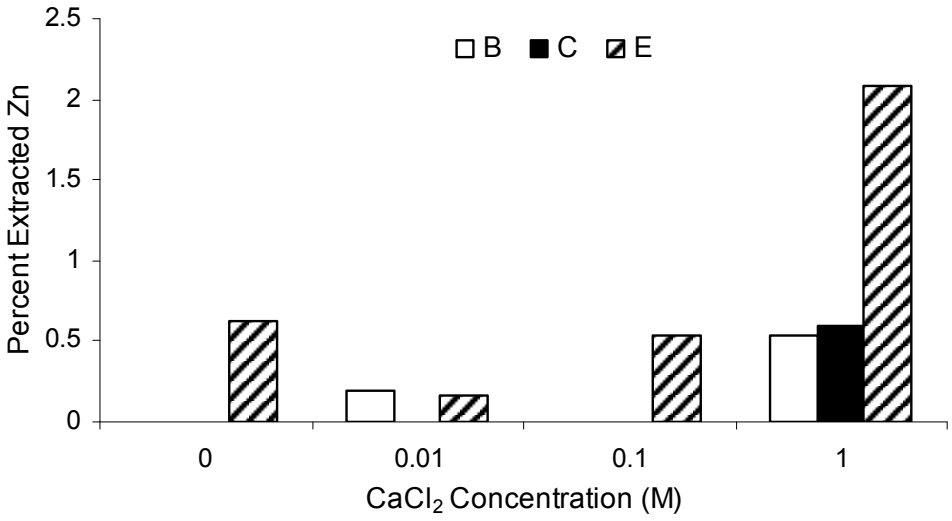


Fig. A.3. Percent of total Zn that was extracted from soils B, C, and E using different concentrations of $CaCl_2$. Where no column is visible, the element was present below detection limits in extracts; *n* =1.

A.1.1. Development of the cyclodextrin extraction

Cyclodextrin has not been used previously to extract metals from contaminated soils. The concentration of cyclodextrin used to extract PAHs and PHCs from soils ranges from approximately 40 g/L to 80 g/L. A concentration of 40 g/L was selected as an ideal extract concentration because it represents the lower range of concentrations used in the literature and requires a relatively low volume of reagents. The potential decrease in efficiency using this concentration was investigated by extracting metals from soil C from Chapter 2 with both a 40 g/L cyclodextrin solution and an 80 g/L cyclodextrin solution for 24 hours. In addition, the impact of extraction time using the 40 g/L solution was investigated by extracting soil C for 0.5, 2, 5, or 24 hours (i.e., the range of times used by other researchers for organic compounds in soil).

For each preliminary extraction, 5 ± 0.01 g of sieved (≤2 mm) air-dried site soil were shaken in cyclodextrin solution in glass centrifuge tubes for 0.5, 2, 5, or 24 hours on a rotary shaker at 120 rpm. The supernatant was removed from each tube using 20-cc disposable syringes and filtered through 0.45 µm cellulose acetate filters into 15-mL centrifuge tubes. Each 10-mL extraction fluid sample was acidified with 0.1 mL of 1 M HCl and stored at 4ºC. Extracts were submitted to ALS Laboratory Group in Waterloo, Ontario, Canada for metal analyses within one week of extraction.

The results of these extractions are provided in Fig. A.4, Fig. A.5, and Fig. A.6. Extracts following 5-hours of extraction contained marginally more Cu, Pb, and Zn than other extracts. Using a higher concentration of cyclodextrin did not appear to increase the concentration of metals in extracts using an extraction time of 24 hours. Therefore, an extraction time of 5 hours and cyclodextrin concentration of 40 g/L was used for the definitive testing.

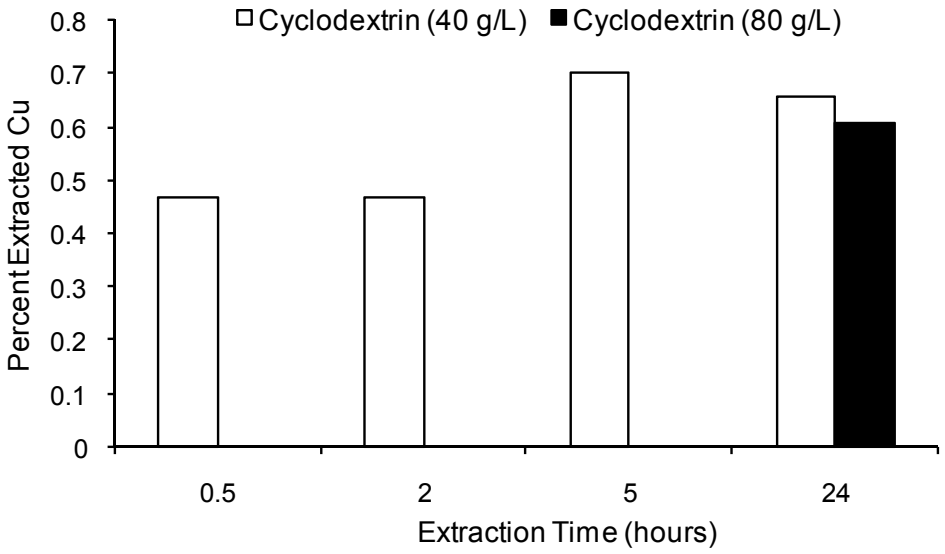


Fig. A.4. Percent of total Cu that was extracted from soil C using different extraction times with 40 g/L cyclodextrin solution. A concentration of 80 g/L was also used for the 24-hour extraction; *n* =1.

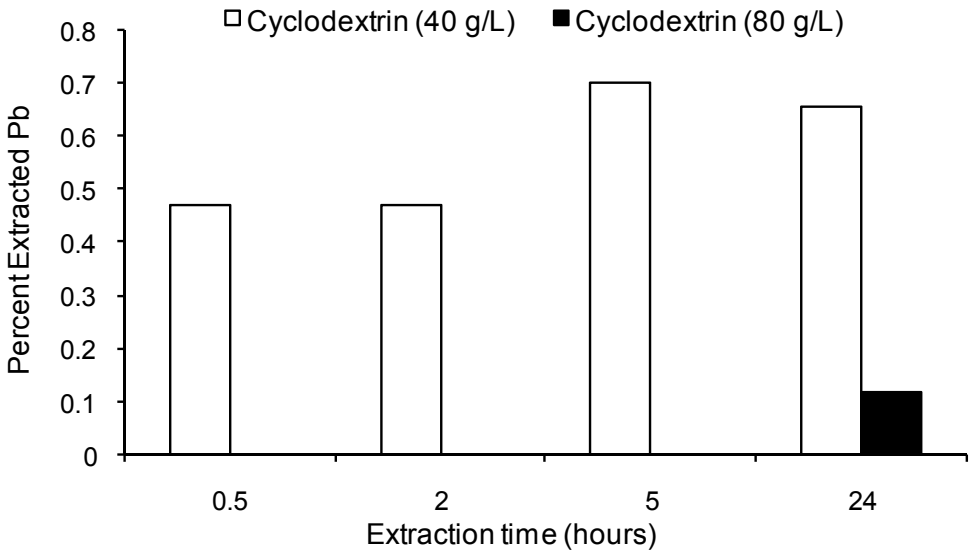


Fig. A.5. Percent of total Pb that was extracted from soil C using different extraction times with 40 g/L cyclodextrin solution. A concentration of 80 g/L was also used for the 24-hour extraction; *n* =1.

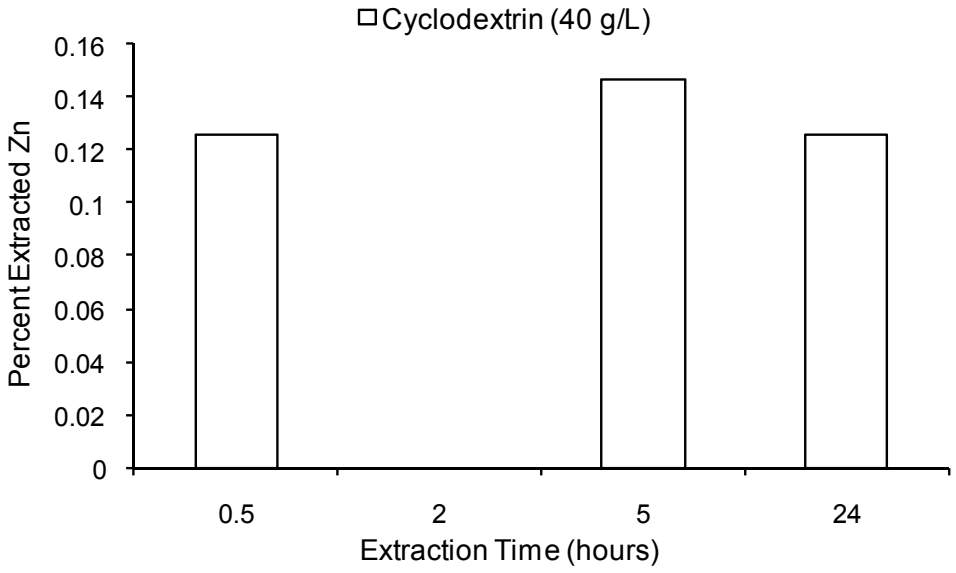


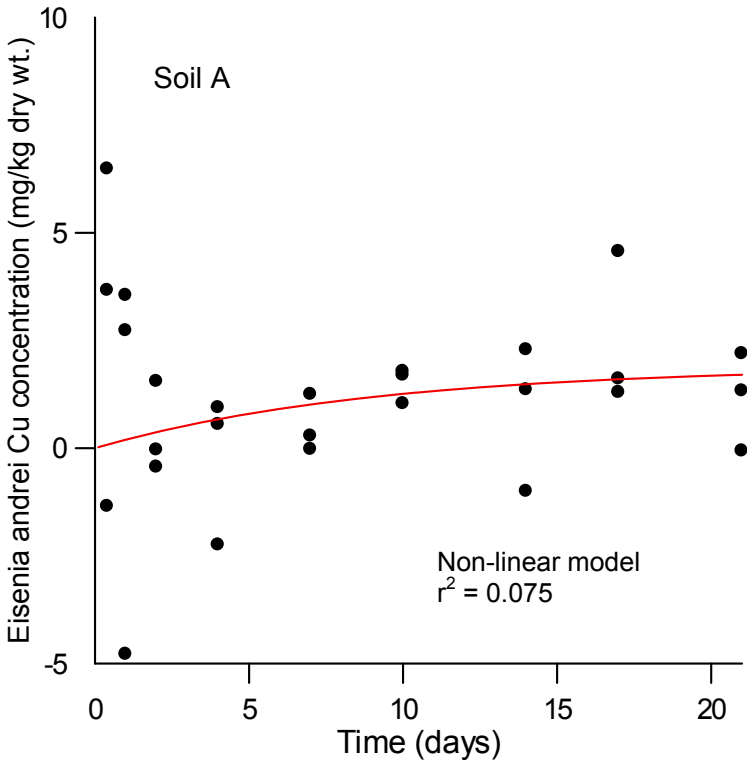
Fig. A.6. Percent of total Zn that was extracted from soil C using different extraction times with 40 g/L cyclodextrin solution. A concentration of 80 g/L was also used for the 24-hour extraction but Zn was not detected in that extract; *n* =1.

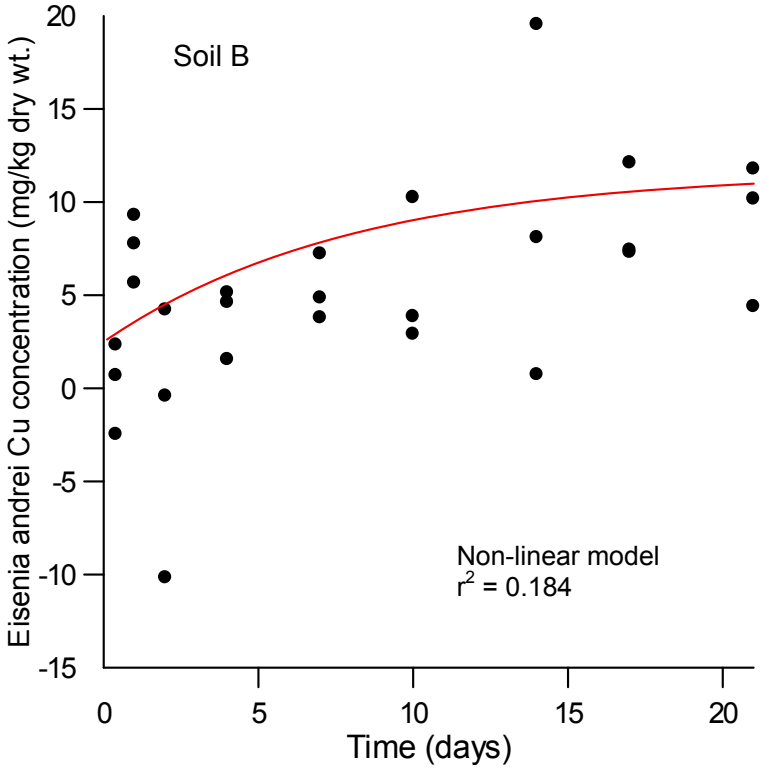
APPENDIX B
EARTHWORM BIOACCUMULATION CURVES

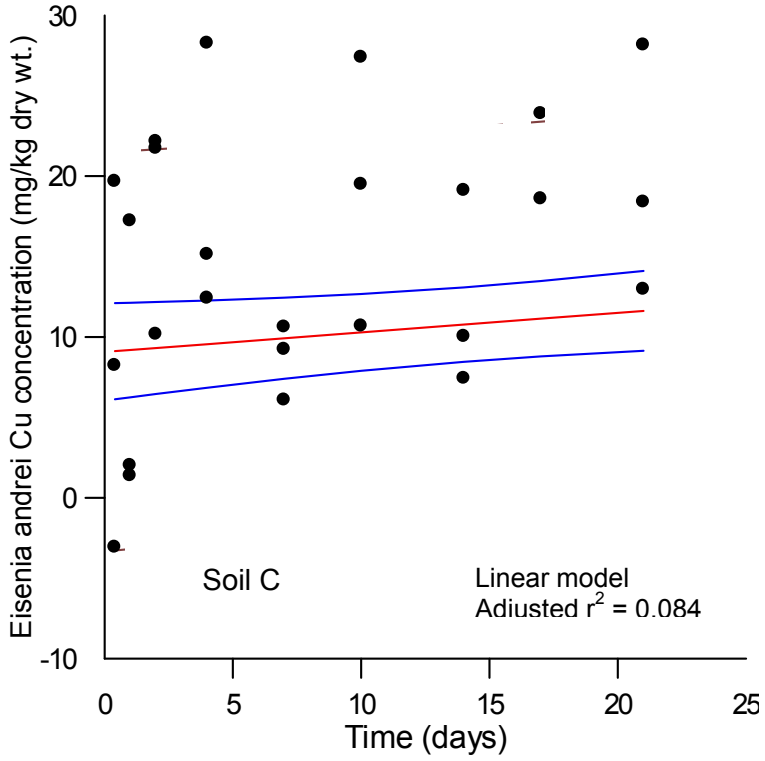
B.1. Bioaccumulation from Chapter 2 soils

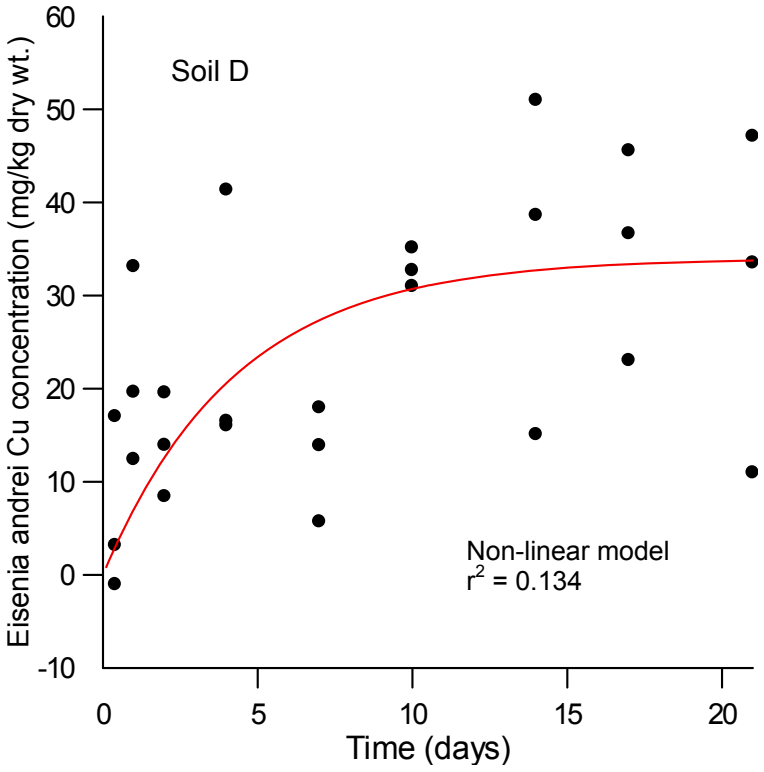
Whole-body concentrations of metal were plotted against time using data from the earthworm bioaccumulation test. Uptake was modeled using linear or non-linear regression procedures (see Chapter 2). The resulting uptake curves are provided within this Appendix. The model which best described the data is illustrated in the graphs herein.

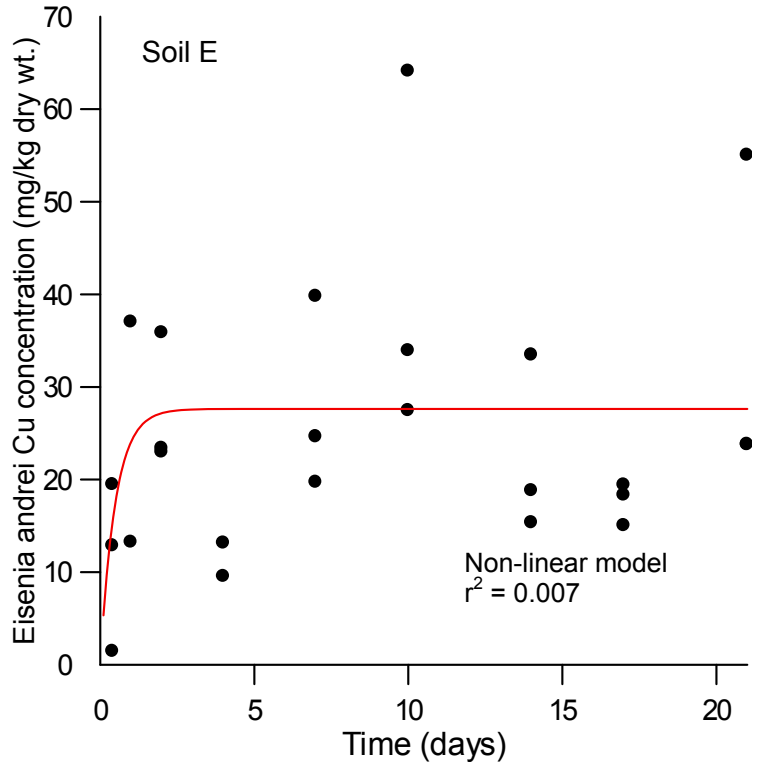
B.1.1. Copper uptake curves



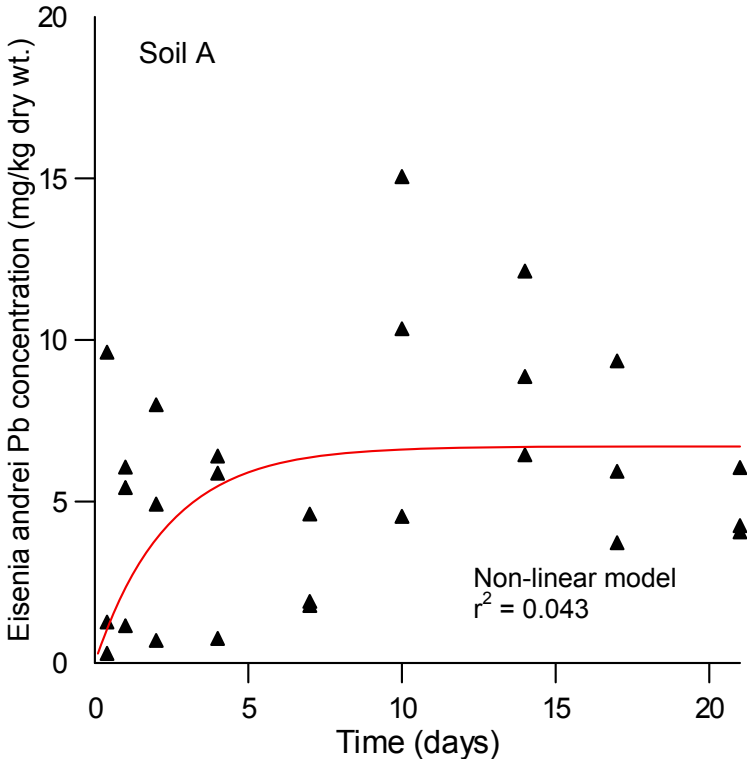

Soil B
Eisenia andrei Cu concentration (mg/kg dry wt.)
Time (days)
Non-linear model
r2 = 0.184
20
15
10
5
0
-5
-10
-15
0
5
10
15
20

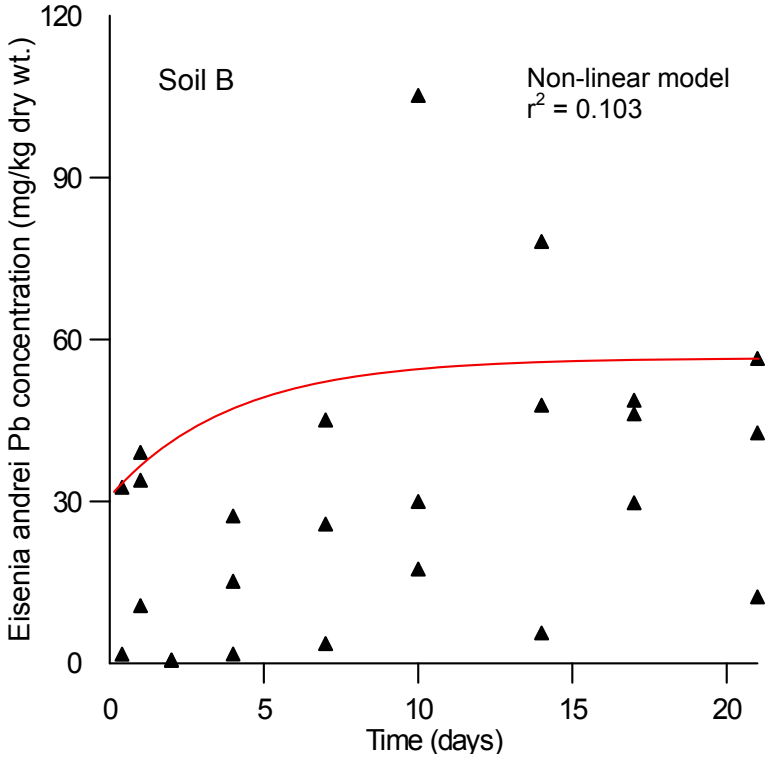

Soil C
Eisenia andrei Cu concentration (mg/kg dry wt.)
Time (days)
Linear model
Adjusted r2 = 0.084
30
20
10
0
-10
0
5
10
15
20
25

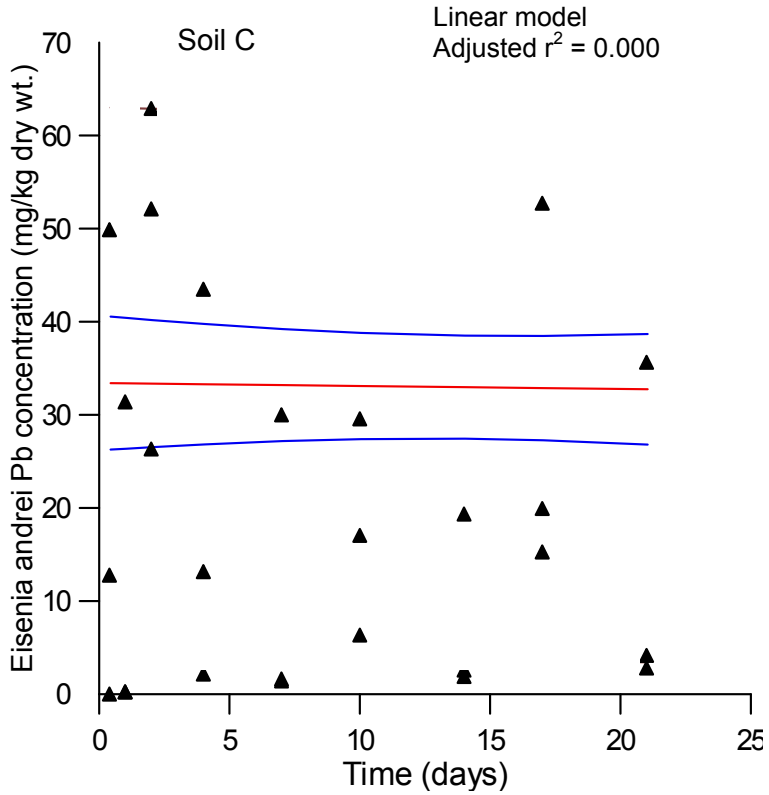

Soil D
Eisenia andrei Cu concentration (mg/kg dry wt.)
60
50
40
30
20
10
0
-10
0
5
10
15
20
Time (days)
Non-linear model
r2 = 0.134

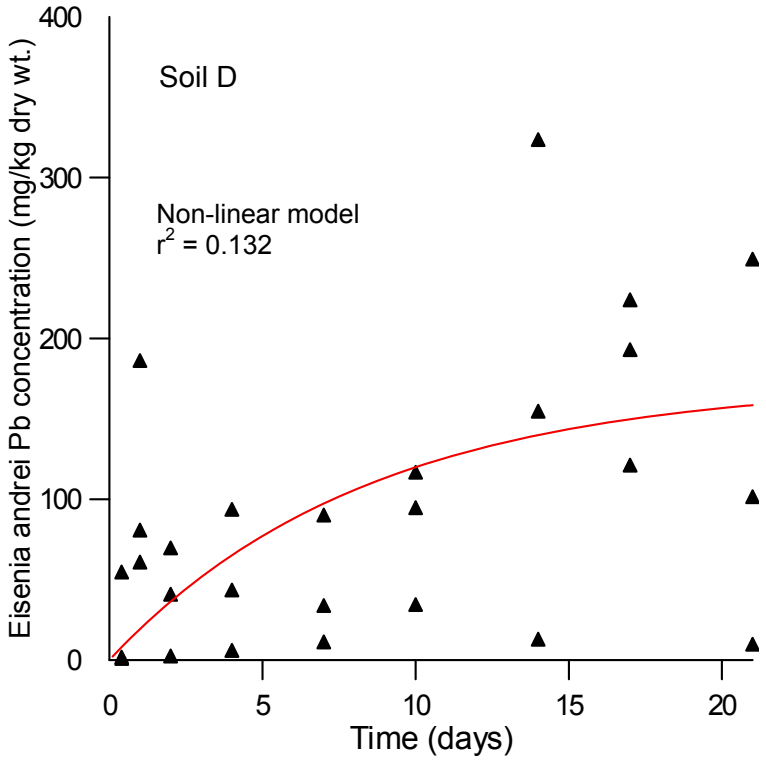

Soil E
Eisenia andrei Cu concentration (mg/kg dry wt.)
70
60
50
40
30
20
10
0
0
5
10
15
20
Time (days)
Non-linear model
r2 = 0.007

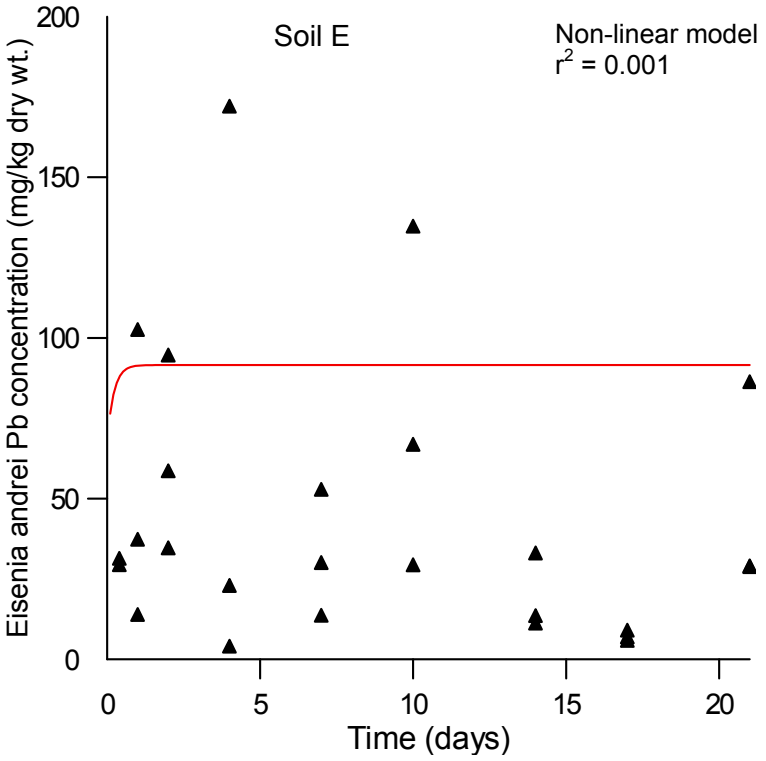
B.1.2. Lead uptake curves



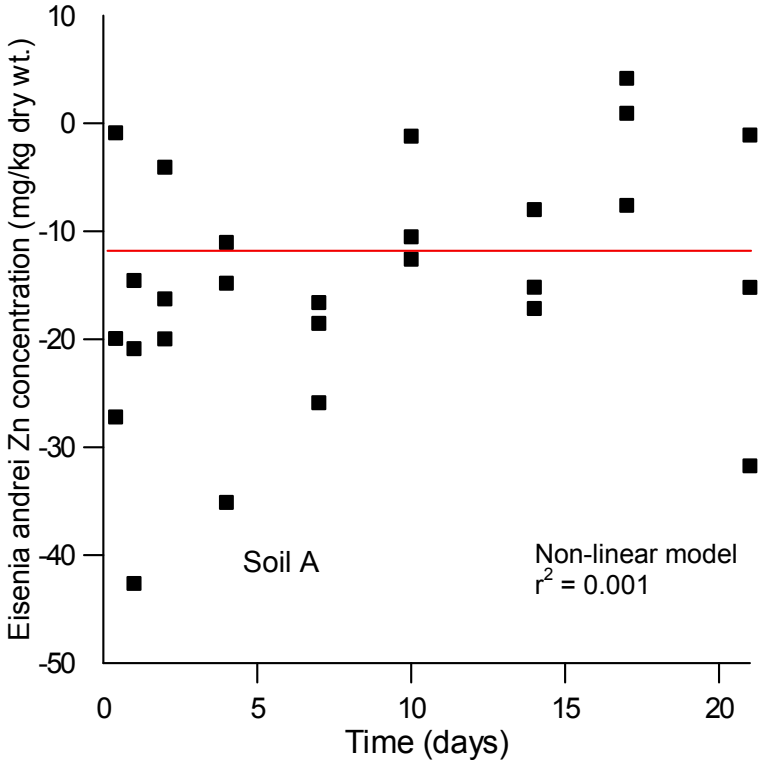

Soil B
Non-linear model
r2 = 0.103
Eisenia andrei Pb concentration (mg/kg dry wt.)
120
90
60
30
0
0
5
10
15
20
Time (days)

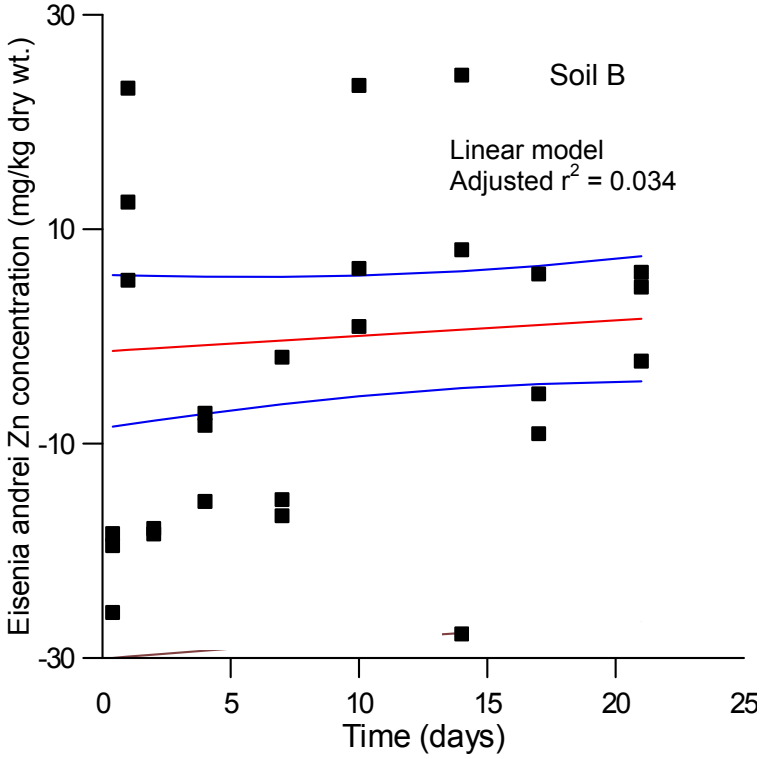

Soil C
Linear model
Adjusted r2 = 0.000
Eisenia andrei Pb concentration (mg/kg dry wt.)
70
60
50
40
30
20
10
0
0
5
10
15
20
25
Time (days)

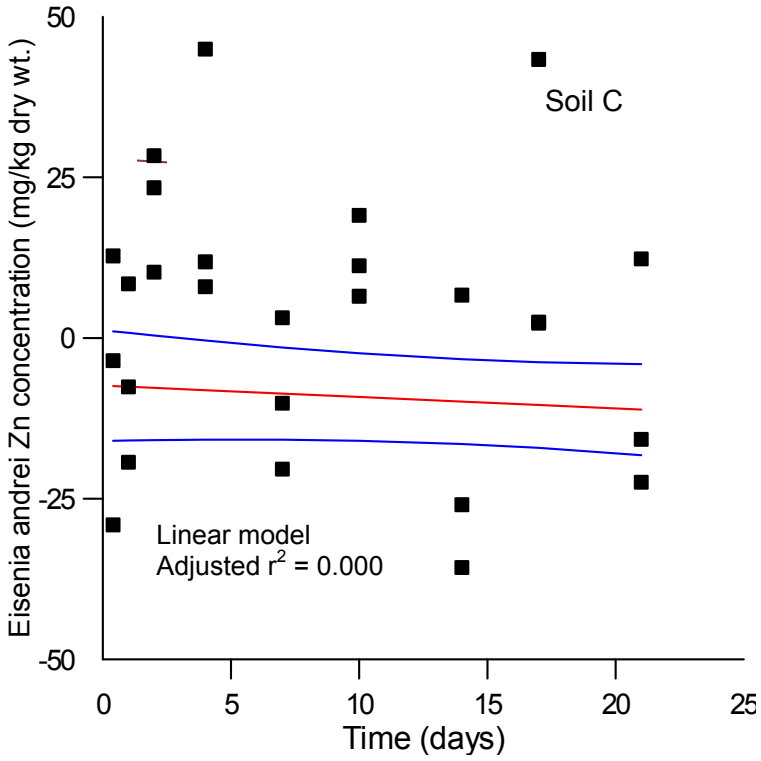

Soil D
Non-linear model
r2 = 0.132
Eisenia andrei Pb concentration (mg/kg dry wt.)
Time (days)
0
100
200
300
400
0
5
10
15
20

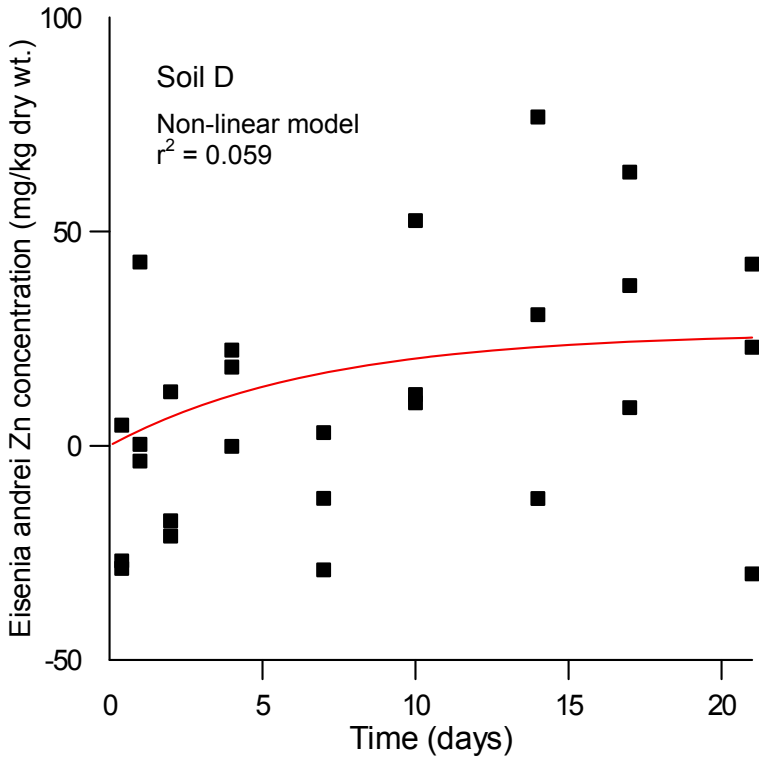

Soil E
Non-linear model
r2 = 0.001
Eisenia andrei Pb concentration (mg/kg dry wt.)
Time (days)
0
50
100
150
200
0
5
10
15
20

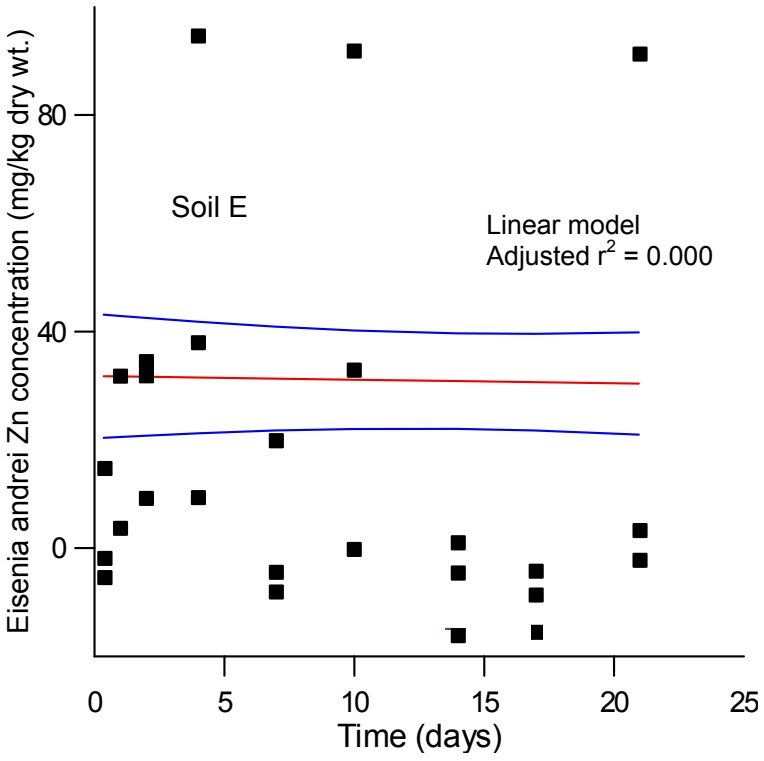
B.1.3. Zinc uptake curves




Soil B
Linear model
Adjusted r2 = 0.034
Eisenia andrei Zn concentration (mg/kg dry wt.)
Time (days)
30
10
-10
-30
0
5
10
15
20
25


Soil C
Linear model
Adjusted r2 = 0.000
Eisenia andrei Zn concentration (mg/kg dry wt.)
Time (days)
50
25
0
-25
-50
0
5
10
15
20
25

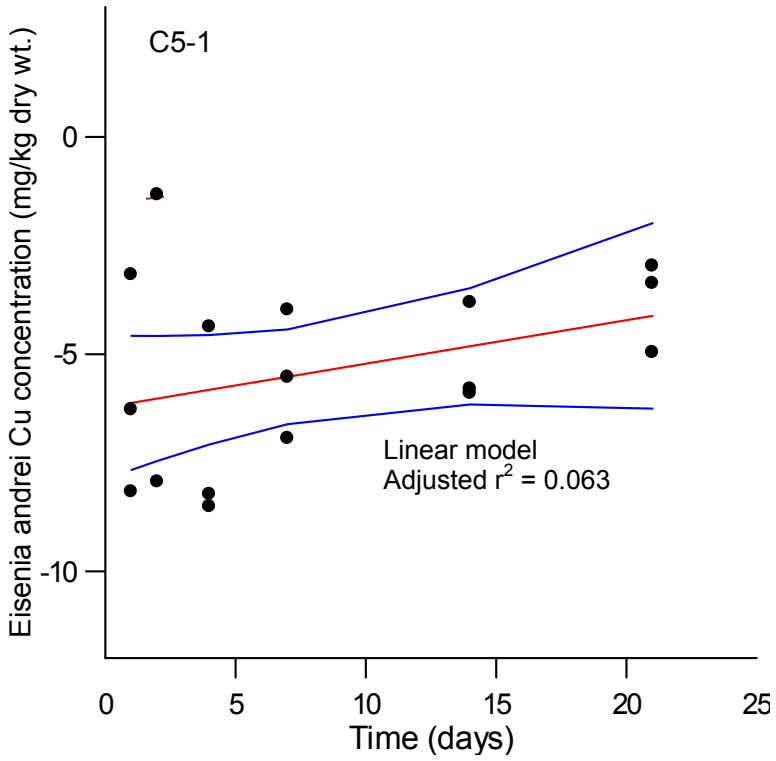

Soil D
Non-linear model
r2 = 0.059
Eisenia andrei Zn concentration (mg/kg dry wt.)
100
50
0
-50
0
5
10
15
20
Time (days)

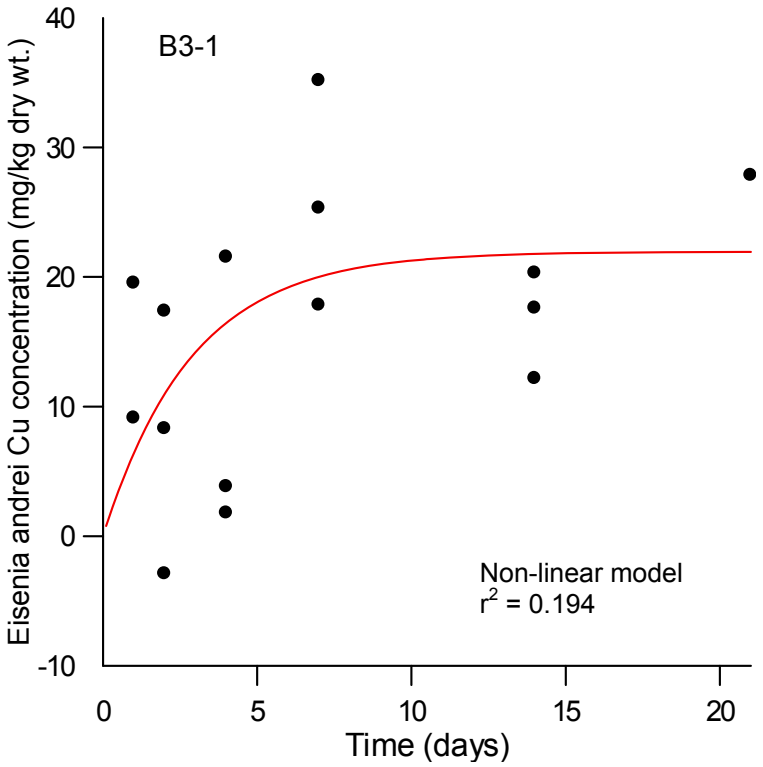

Soil E
Linear model
Adjusted r2 = 0.000
Eisenia andrei Zn concentration (mg/kg dry wt.)
80
40
0
0
5
10
15
20
25
Time (days)

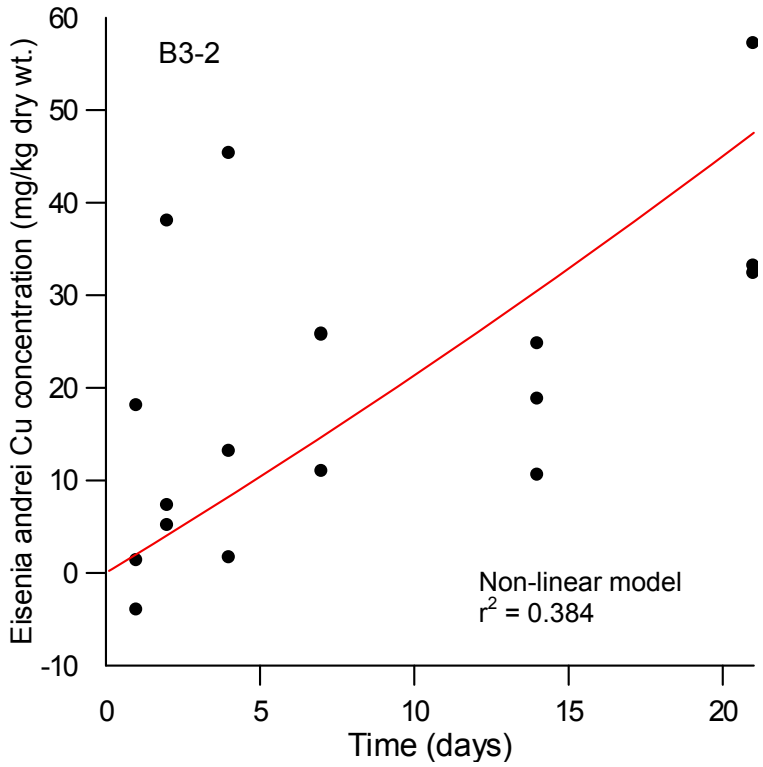
B.2. Bioaccumulation from Chapter 3 soils

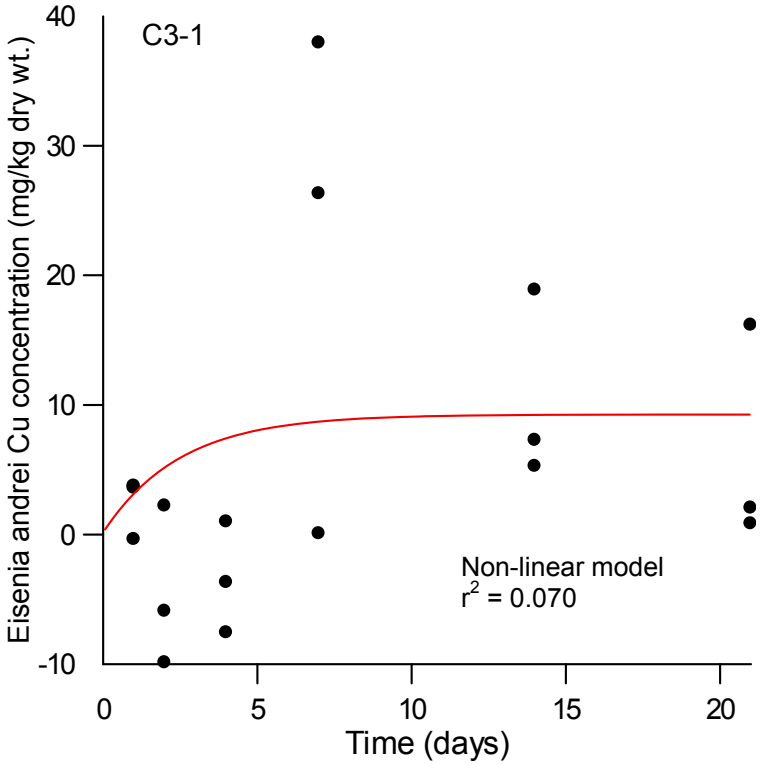
Whole-body concentrations of metal were plotted against time using data from the earthworm bioaccumulation test. Uptake was modeled using linear or non-linear regression procedures (see Chapter 3). The resulting uptake curves are provided within this Appendix. The model which best described the data is illustrated in the graphs herein.

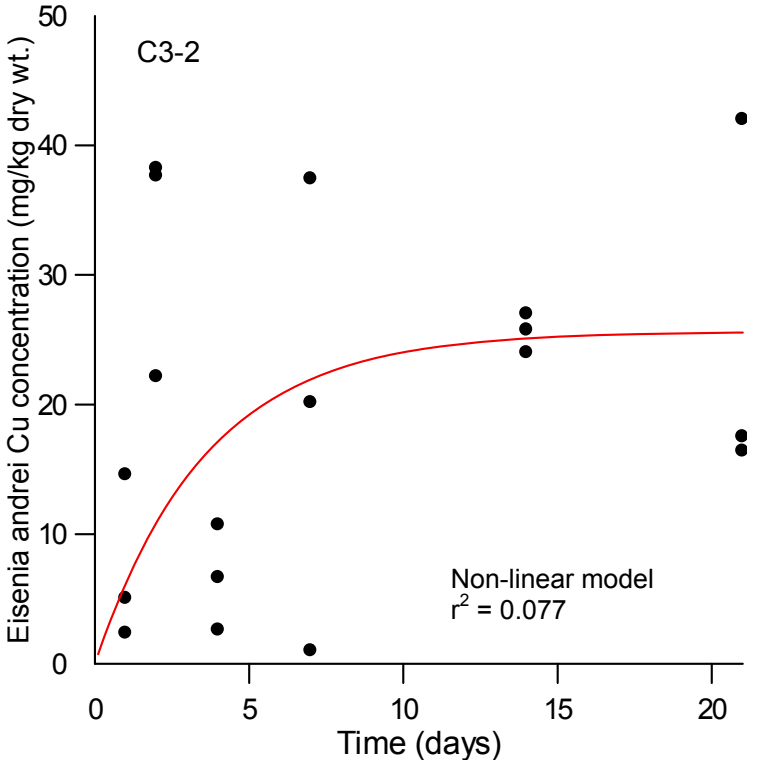
B.2.1. Copper uptake curves

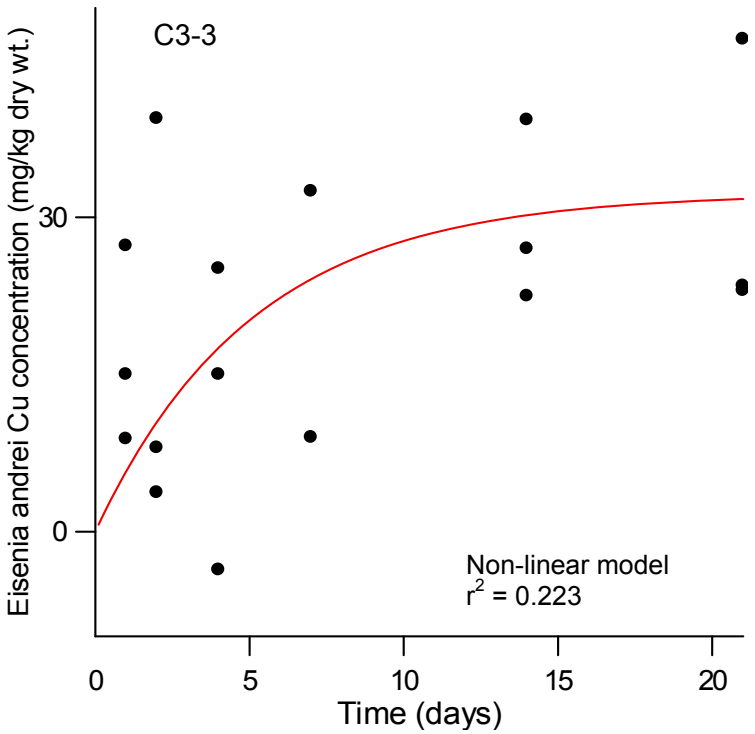



B3-1
Eisenia andrei Cu concentration (mg/kg dry wt.)
40
30
20
10
0
-10
0
5
10
15
20
Time (days)
Non-linear model
r2 = 0.194

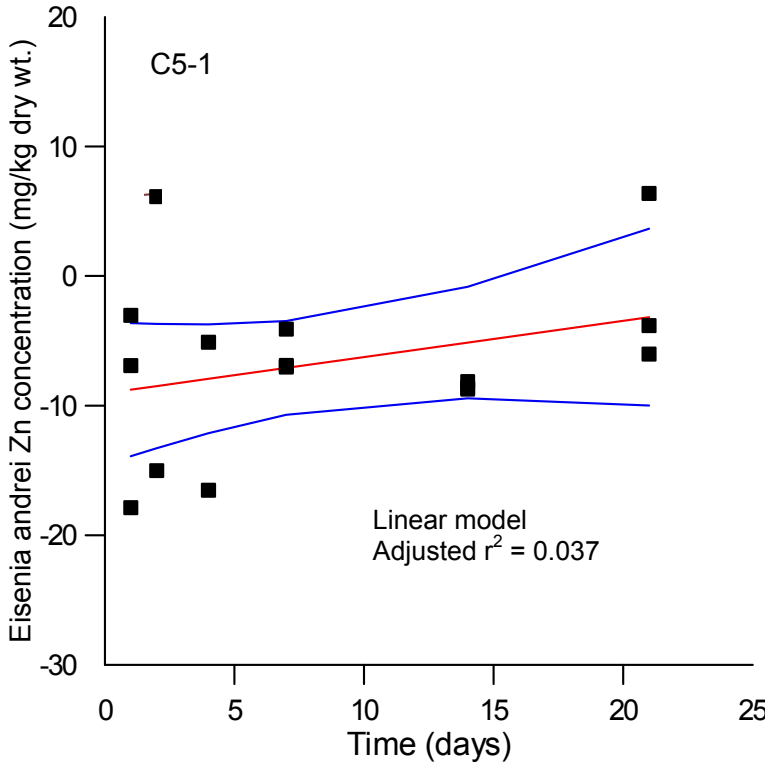

B3-2
Eisenia andrei Cu concentration (mg/kg dry wt.)
60
50
40
30
20
10
0
-10
0
5
10
15
20
Time (days)
Non-linear model
r2 = 0.384

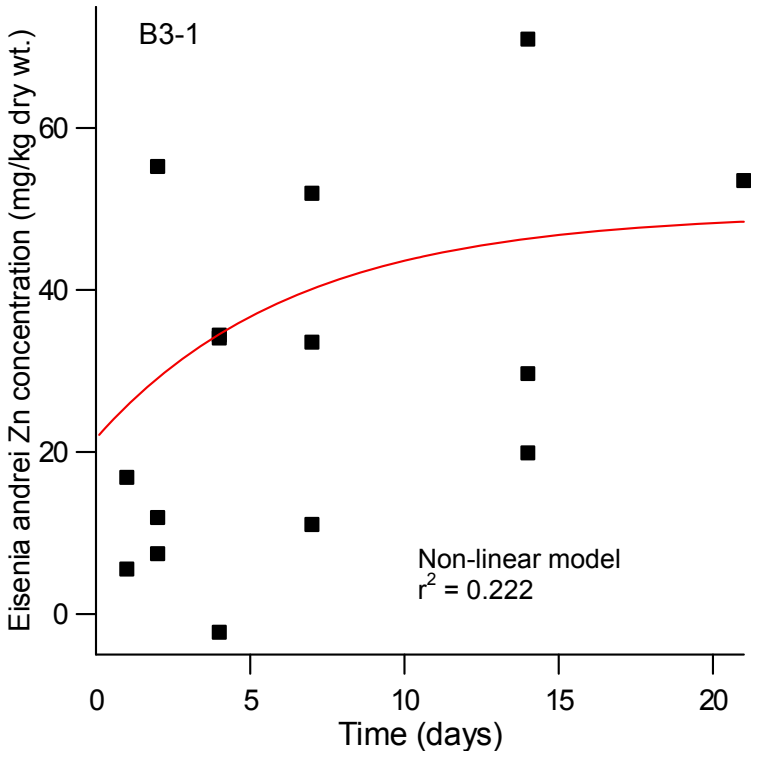

C3-1
Eisenia andrei Cu concentration (mg/kg dry wt.)
40
30
20
10
0
-10
0
5
10
15
20
Time (days)
Non-linear model
r2 = 0.070

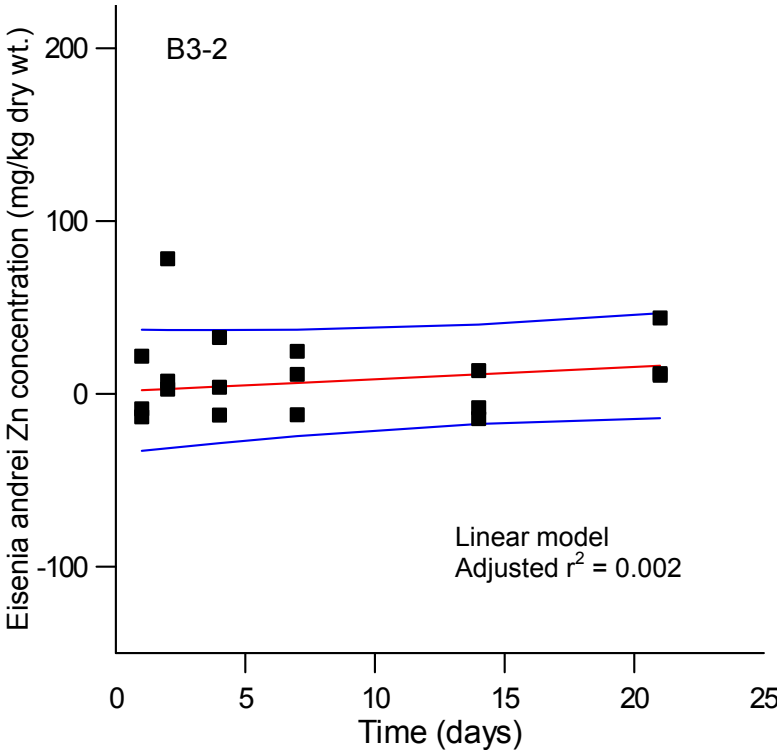

C3-2
Eisenia andrei Cu concentration (mg/kg dry wt.)
50
40
30
20
10
0
0
5
10
15
20
Time (days)
Non-linear model
r2 = 0.077

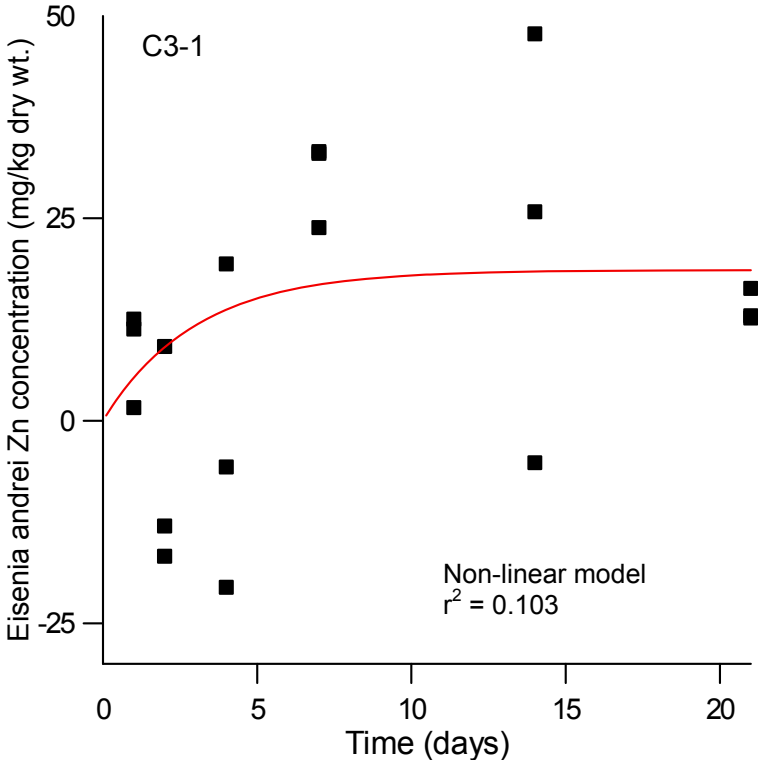

C3-3
Eisenia andrei Cu concentration (mg/kg dry wt.)
30
0
Non-linear model
r2 = 0.223
0
5
10
15
20
Time (days)

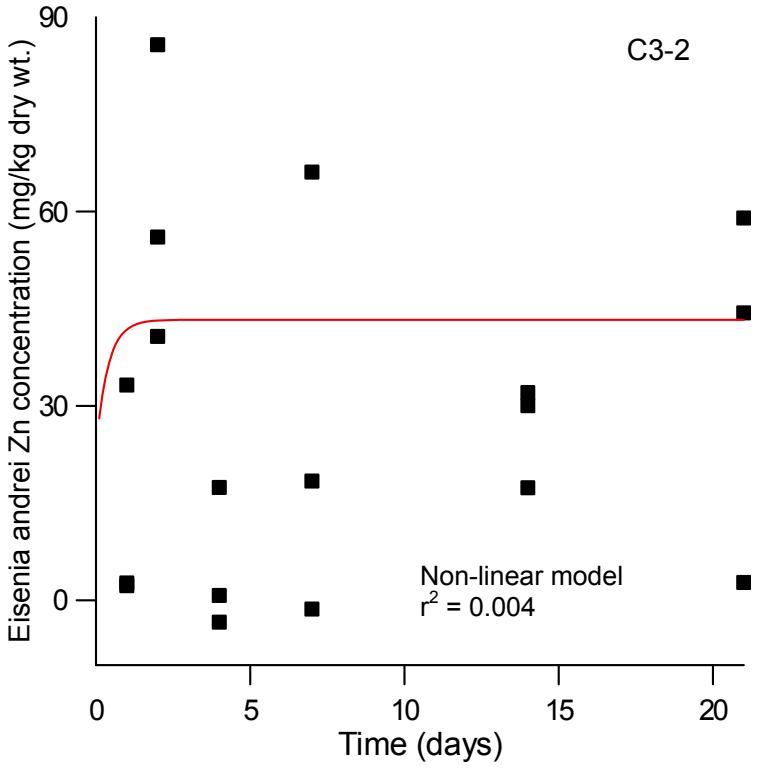
B.2.2. Zinc uptake curves

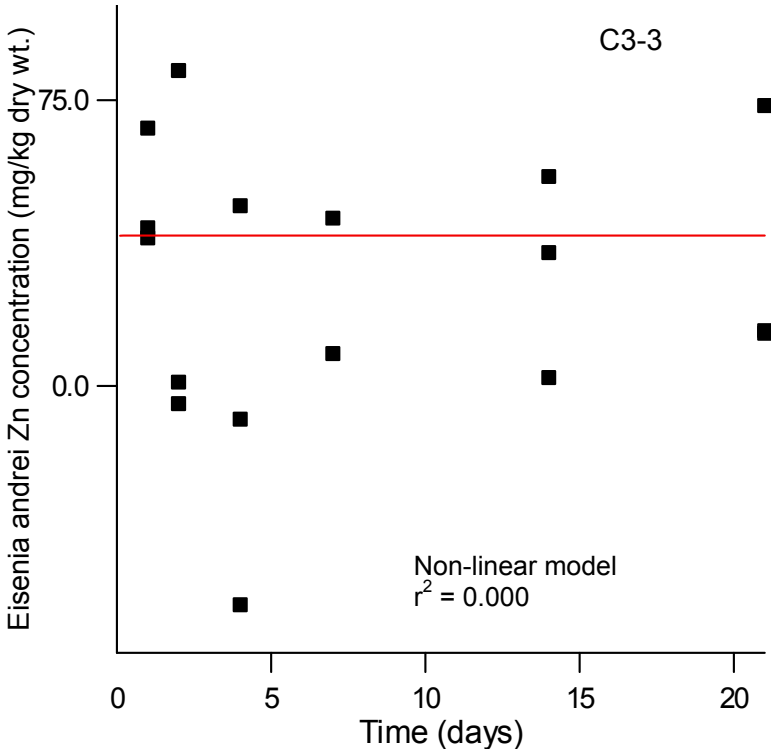



B3-1
Eisenia andrei Zn concentration (mg/kg dry wt.)
60
40
20
0
0
5
10
15
20
Time (days)
Non-linear model
r2 = 0.222


B3-2
Eisenia andrei Zn concentration (mg/kg dry wt.)
200
100
0
-100
0
5
10
15
20
25
Time (days)
Linear model
Adjusted r2 = 0.002


C3-1
Eisenia andrei Zn concentration (mg/kg dry wt.)
50
25
0
-25
0
5
10
15
20
Time (days)
Non-linear model
r2 = 0.103


C3-2
Eisenia andrei Zn concentration (mg/kg dry wt.)
90
60
30
0
0
5
10
15
20
Time (days)
Non-linear model
r2 = 0.004


C3-3
Eisenia andrei Zn concentration (mg/kg dry wt.)
75.0
0.0
Non-linear model
r2 = 0.000
0
5
10
15
20
Time (days)